

Sex-based differences in IMCL storage and utilization during an acute bout of high intensity
interval exercise (HIIE)

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

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Abstract

INTRODUCTION: The contribution of lipids, particularly intramyocellular lipids (IMCL), to energy production during high intensity interval exercise (HIIE) is not well characterized. Sex influences lipid utilization during moderate intensity aerobic exercise and thus there may be differences in lipid utilization during HIIE as well. There is conflicting evidence as to whether there is a sex difference in IMCL utilization during exercise, which may be a result of improper matching, differences in methodology, or differential use in subcellular regions. **PURPOSE:** Our study aimed to determine whether IMCL are used during a bout of HIIE and whether sex influences the extent of IMCL utilization. We also examined whether sex or exercise influenced IMCL storage characteristics and morphology. **METHODS:** Young, healthy, recreationally active males (n=12) and females (n=12) were matched for aerobic fitness ($\text{mL O}_2 \cdot \text{kgFFM}^{-1}$) and performed an acute bout of HIIE (5 min warm up/cooldown @50W, 10 x 1-min @90% HRmax). Biopsies were collected from the *vastus lateralis* prior to and following exercise for electron microscopic determination of IMCL storage and western blot analysis of proteins related to lipid and aerobic metabolism. **RESULTS:** Carbohydrate oxidation ($p < 0.0001$) and the increase in blood lactate ($p = 0.02$) were greater in males than females during HIIE, with no difference in fat oxidation ($p = 0.98$). Sex had no effect on content of any proteins analyzed (all $p > 0.08$) or on IMCL area density (all $p > 0.31$). IMCL area density did not change during exercise (all $p > 0.37$) and there was no difference in utilization between the sexes (all $p > 0.47$). IMCL feret ($p < 0.001$), perimeter ($p < 0.004$) and average length of IMCL-mitochondria apposition (all $p < 0.03$) were greater in males. IMCL number ($p < 0.03$) and perimeter to area ratio ($p < 0.006$) were greater in females. **CONCLUSION:** Fat and IMCL are not significant contributors to energy production during HIIE. Comparable rates of fat oxidation and a lesser increase in blood lactate in females suggest that females relied on anaerobic glycolysis to a lesser extent. Excitingly, our region-

specific characterization of IMCL morphology establishes a basis for sex-difference investigation into IMCL storage characteristics.

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Dedication

For my grandparents. Mama, Yeye, Popo, and Gonggong. I am so lucky to have been able to spend these years knowing and losing in Mahjong to you all. It's not lost on me that everything I have today would not be if not for the decisions you all made. I hope that I've made you all proud, and will continue to work to do so (and to maintain my position as your favourite grandchild). I love you all so much.

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List of Abbreviations

Abbreviation	Unabbreviated Form
T2D	Type II Diabetes Mellitus
HEPT	High Energy Phosphate Transfer
VO ₂ max	Maximal oxygen consumption
HIIE	High Intensity Interval Exercise
HIIT	High Intensity Interval Training
SIE	Sprint Interval Exercise
SIT	Sprint Interval Training
MICE	Moderate Intensity Continuous Exercise
MICT	Moderate Intensity Continuous Training
IMCL	Intramyocellular lipids
EMCL	Extramyocellular lipid droplet
SS	Subsarcolemmal
FA/FFA	Fatty Acid/Free Fatty Acid
LB FFM/FFM	Lower Body Fat Free Mass/ Fat Free Mass
IMF	Intermyofibrillar Region
TEM	Transmission Electron Microscopy
RPE	Rating of Perceived Exertion
HR	Heart Rate
CHO	Carbohydrates
TAG	Triacylglycerol
DAG	Diacylglycerol
MAG	Monoacylglycerol
H-MRS	Proton magnetic resonance spectroscopy
ATP	Adenosine Triphosphate
TEE	Total Energy Expenditure
SEM	Standard Error of the Mean

Chapter 1: Introduction and Literature Review

1.0 General Introduction

High intensity interval exercise (HIIE) is a popular mode of exercise involving repeated short duration high intensity intervals interspersed with low-intensity/rest intervals of equal duration¹. HIIE has become popular because it can induce comparable benefits to moderate intensity continuous exercise (MICE)²⁻⁶ despite a reduced exercise time. Given the high intensity nature of the HIIE bout, carbohydrates(CHO) are the main source of fuel used to support exercise energy needs^{7,8}; however, given that half of the exercise bout is spent at low intensity/rest, lipids may also contribute substantially to adenosine triphosphate (ATP) production. Furthermore, investigation of substrates contributing to ATP production have largely omitted the sources and contribution of lipids. This may be due to the conception that energy demand during HIIE is mainly supplied by anaerobic energy systems due to the higher intensities of the work intervals⁷. However, the lower overall ‘average’ intensity may suggest a more significant contribution of aerobic systems than what is currently considered. Combined with well-established evidence that lipid sources contribute significantly toward aerobic energy production⁹⁻¹¹, and positive adaptations to the cardiorespiratory system with high intensity interval training (HIIT)¹, deeper investigation may be warranted on lipid sources during HIIE. Alongside plasma free fatty acids (FFA), intramyocellular lipids (IMCL) are an important contributor to fuel utilization during moderate intensity continuous exercise (MICE)⁸. The contribution of each vary due to a number of factors, including intensity and exercise duration⁸. To our knowledge, whether the contribution of IMCL remains significant when extending past endurance exercise to HIIE has not been investigated. Further, sex differences have been well established in endurance exercise¹¹⁻¹⁵ with females exhibiting a higher reliance on lipids as a fuel source. Despite this, in

humans, the contribution of IMCL to lipid oxidation during endurance exercise is discordant^{13,16–20}. This may be owed to differences in sensitivity of testing methods, however, prevailing gold standards of proton magnetic resonance spectroscopy (H-MRS) and transmission electron microscopy (TEM) produce results largely in agreement with one another^{10,21}. Utilizing TEM to analyze both total and localized IMCL utilization across the sexes for an acute bout of HIIE may then shed light upon an area that has been left relatively untouched in the literature.

1.1 Fuel Utilization During Exercise

1.1.1 Predominating Energy Pathways during Exercise

During exercise ATP is needed for muscle contraction to occur. Skeletal muscle stores of ATP are small and can only provide ATP for the first ~2 seconds of exercise²². As such, skeletal muscle has several pathways that can be used to produce ATP. These pathways include the high energy phosphate transfer (HEPT), glycolysis, β -oxidation, the Krebs cycle, and oxidative phosphorylation. These pathways are further classified as being anaerobic (HEPT, glycolysis) or aerobic (β -oxidation, Krebs cycle, oxidative phosphorylation). Contrary to the classical energy continuum proposal, by which the ‘turning off’ of one energy system coincides with a ‘turning on’ of the next, evidence has shown that all pathways are activated at the onset of exercise⁷. The relative contribution of each pathway, however, differs with variables corresponding to the individual and the exercise bout. The dominance of each pathway is predicated by training status^{23–26}, sex^{12,13,16,19,20,27–29}, exercise intensity^{8,22,30,31} and duration^{8,22}, among other factors. Notably, during prolonged exercise aerobic metabolism predominates with the majority of ATP being produced from the oxidation of CHO and fats.

1.1.2 CHO and fat oxidation during exercise - influence of exercise duration and intensity

Energy requirements are met through breakdown of CHO, fats, and protein²². During exercise, the contribution of protein to energy expenditure is minimal (~2-5%) in comparison to CHO and fats, which are the primary substrates²². Sources of fat used during exercise include adipose triglycerides and skeletal muscle intramyocellular lipids (IMCL). Sources of carbohydrate used during exercise include hepatic and skeletal muscle glycogen. The type and source of substrate used to support exercise energy needs is dependent on diet and training status, as well as intensity and duration of the activity⁸. Generally, fats are the preferred source at rest and at low-to-moderate exercise intensities^{8,17}, whereas CHO become the predominant source at higher intensities^{8,32} and fat oxidation declines at exercise intensities above ~65% VO_{2max}⁸. Looking at the source of fat oxidation during exercise, at lower exercise intensities plasma free fatty acid (FFA) oxidation predominates, with an increased reliance on FFA from IMCL lipolysis with increasing exercise intensities⁸. With respect to CHO oxidation there is an increase in oxidation of glucose from both hepatic and muscle glycogen stores with increased exercise intensity, with the contribution from muscle glycogen increasing to a greater extent than that of hepatic glycogen⁸. Exercise duration also serves as a determining factor in fuel preference, with preference shifting from CHO to fats with increased duration⁸. Furthermore, while at the onset of exercise muscle and plasma sources of fuel contribute similarly to energy production, with increasing exercise duration muscle sources contribute less and plasma sources predominate⁸.

1.1.3 Why fat oxidation is limited at higher exercise intensities

As discussed previously, it is widely accepted that the contribution of lipids to ATP production decreases at exercise intensities above ~65%VO_{2max}^{7,30,31}, which can be noted in Figure 1.1. Early studies suggested that this shift was owing to a decrease in adipocyte lipolysis³³, however, widely recognized reports from Romijn et al^{34,35}, have contested this. While

their 1993 inquiry noted a decrease in plasma FFA concentration at an exercise intensity of 85% $\text{VO}_{2\text{max}}$, the rate of lipolysis as estimated by the rate of glycerol appearance was not lower at this intensity compared to 65% $\text{VO}_{2\text{max}}$ ³⁴. This same group later investigated whether the infusion of lipids would offset the differences in lipid oxidation observed at 85% $\text{VO}_{2\text{max}}$ and 65% $\text{VO}_{2\text{max}}$ ³⁵. Interestingly, while lipid oxidation was elevated with infusion at 85% $\text{VO}_{2\text{max}}$, it was still markedly below rates observed in the 65% $\text{VO}_{2\text{max}}$ exercise bout³⁵. Furthermore, while the study by Christmass et al. reported decreased rates of lipid oxidation during sprint interval exercise (SIE), plasma glycerol was also not different compared to the bout of continuous exercise, which was matched for intensity as a percentage of $\text{VO}_{2\text{peak}}$ ³⁶. Early evidence showed that lactate negatively affects the release of FFA resulting from adipocyte lipolysis³⁷. Newer studies, however, have suggested that not only is the release of FFA impaired by lactate, but also have reported an anti-lipolytic effect of lactate^{38–40}. Lactate is released from skeletal muscle as a by-product of glycolytic flux during intense exercise or periods of oxygen deficit^{39–41}. A notable study by Boyd et al found an inhibitory effect of lactate on plasma concentrations of FFA and glycerol in exercising humans⁴². More recently, studies have shown that the anti-lipolytic effect may be through action on a G-protein coupled receptor (GPCR), GPR81^{38–40}. Liu et al reported activation of GPR81 by lactate, followed by an internalization, and negative regulation of adenylate cyclase^{39,40}. Adenylate cyclase acts to increase cAMP, which through interaction with PKA will downstream stimulate release of FFA from lipolysis³⁹. Together, these findings suggest that, in part, the observed decrease in plasma FFA concentration during high intensity exercise may be due to both a decrease in adipocyte lipolysis as well as impaired release of FFA into the plasma. Furthermore, alterations in blood flow during higher intensity exercise can also reduce delivery of FFA to the active muscle tissue^{33,37–40}.

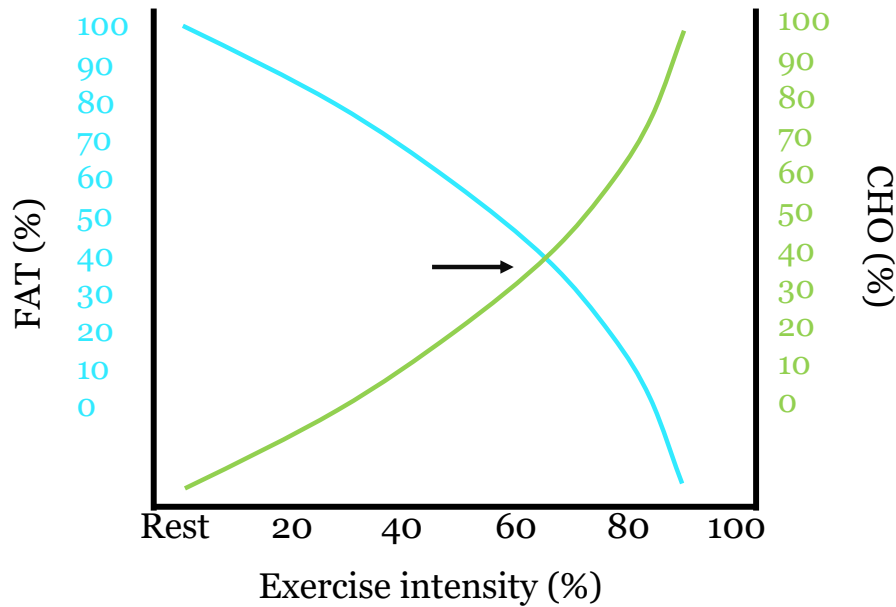


Figure 1.1. A schematic depicting the classic crossover pattern observed between fuel utilization and exercise intensity. Adapted from Brooks et al (1994)⁴³.

Downstream of the delivery of FFA to the active tissue via routing of blood flow, additional steps are required for FFA to enter muscle cells and further, mitochondria, for oxidation³³. Fatty acids enter the muscle through passive diffusion or by utilizing fat transporters (CD36, FABP_{pm}, FATP1/FATP4)^{44,45}. Once inside the myocyte the fatty acid is linked to coenzyme A (CoA) in the cytosol and targeted either towards storage or oxidation⁴⁵. If fated for oxidation, long-chain fatty acyl-CoAs are transported into the mitochondria via the carnitine shuttle where the first step of this process, the substitution of carnitine for the CoA, catalyzed by carnitine palmitoyl transferase 1 (CPT1) is the rate limiting step of fat oxidation⁴⁵. After successfully entering the mitochondrial matrix, the FA-CoA undergoes beta-oxidation⁴⁵. Interestingly, while long chain fatty acids require the carnitine shuttle to cross the inner mitochondrial membrane, shorter and medium chain fatty acids are able to diffuse into the mitochondria to undergo beta-oxidation^{46,47}. Further, evidence has shown a decrease in the rate of oxidation of long chain, but not medium chain fatty acids when infused during an exercise bout corresponding to an intensity of 80%

VO_{2max} compared to at 40% VO_{2max}⁴⁸. Thus, it has been suggested that increasing exercise intensity also hinders the potential for transport of long-chain fatty acids into the mitochondria, which is thought to be due to inhibition of CPT-1⁴⁶. As such, the two main hypothesized reasons as to why fat oxidation decreases during higher intensity exercise are decreased delivery of FFA to the muscle cell itself and/or decreased FFA entry into the mitochondria for oxidation by CPT1.

1.1.4 Interval Exercise

1.1.4.1 Defining HIIE and SIE

HIIE and sprint interval exercise (SIE) are two main forms of interval exercise¹. They are both forms of aerobic exercise involving short periods of high intensity exercise interspersed with periods of reduced intensity or rest. The differences lie wherein the high intensity intervals in HIIE generally refers to intense but submaximal exercise at approximately 80 to ~100% VO_{2max}^{1,49}. By contrast, the high intensity intervals in SIE involve intensities expected to correspond to VO_{2max} or greater and may be referred to as “all-out” or supramaximal efforts^{1,49}. Furthermore, the two generally differ by work-to-rest ratio, in that in HIIE the high and rest/low intervals are usually similar in length (1:1) whereas in SIE the low/rest intervals are longer in duration relative to the high intensity interval (~1:3/4)⁵⁰.

1.2 Fuel Utilization and Metabolism during HIIE

Despite growing evidence that supports interval training as an effective method for increasing cardiorespiratory and skeletal muscle fitness as well as improving insulin sensitivity and body composition^{2,4-6,50}, substantially less investigation has been performed to characterize the patterns by which ATP demand is met during HIIE compared to what is known about fuel metabolism during MICE. With respect to each work effort generally lasting up to 60 seconds,

and through repeated cycles, ATP demands are met through the same three aforementioned energy systems as has been well studied in MICE⁸. However, while ATP is produced predominately through aerobic systems during MICE, it has been estimated that energy demands for bouts lasting roughly 60 seconds at a high intensity are met primarily through anaerobic systems, with an approximate 30% contribution from aerobic systems⁸.

Intensities corresponding to the work efforts required during HIIE occur after the ‘crossover point’ by which CHO overtake fats as the preferred fuel source to support ATP production^{1,8,22}. As a consequence to this, and in part due to the aforementioned “energy continuum” mentioned in 1.1.1, there lies a fallacy in which total production of ATP during an acute bout of HIIE is supplied through the anaerobic systems HEPT and anaerobic glycolysis⁷. This assumption implies a negligible input and importance of the contributions of aerobic systems. However, while anaerobic systems contribute significantly to energy production early into each work effort and acute bout, the importance of oxidative metabolism increases alongside exercise duration, and with varying ratios of work to rest efforts⁷. A widely referenced study regarding fuel utilization and metabolism during HIIE focused largely on anaerobic power output and metabolite data in males performing 10 six-second sprints interspersed with rest periods of thirty seconds⁵¹. At the final work effort, the group reported a decrease in peak power output, as well as a reduction in glycogenolysis despite a notable failure to replenish PCr stores within the recovery period⁵¹. These two findings combined suggest a shift of relative contributions from anaerobic energy systems toward oxidative metabolism as the exercise bout advances^{7,51}. This proposed shift in energy systems remains even in consideration that this exercise bout totalled roughly 6 minutes in length with all-out work efforts. While contributions of oxidative pathways were not investigated, studies that followed have reported increased oxygen consumption with

intermittent exercise at high intensities, both at the level of the whole body⁵², and localized to the active muscle⁵³. These findings support the notion suggested by the Gaitanos et al study⁵¹, of increased importance of oxidative pathways throughout the duration of the HIIE/SIE bout.

Furthermore, the mechanisms by which the varying energy systems predominate during HIIE has been investigated in a trial by Parolin et al.⁵⁴, by which six male participants completed 3 thirty-second all-out sprints, with four-minute rest periods following each work-period. Muscle biopsies were collected at the start and end of the first and last sprints, as well as at 6 seconds and 15 seconds, also during bouts one and three⁵⁴, allowing for profiles of enzyme activity and metabolites to be analyzed. While pyruvate and lactate were observed to increase in the muscle across the first sprint, notably, this was not observed during the third sprint⁵⁴. These results were found alongside a reduction in glycogenolysis and glycogen utilization⁵⁴ when comparing the final and initial sprints. Additionally, as observed in the study by Gaitanos et al⁵¹, there was a decrease in power output with successive sprints. Furthermore, during the third sprint dominance of anaerobic glycolysis to energy production had shifted to oxidative phosphorylation as evidenced by an increase in PDH activity⁵⁴. While the ATP produced to support these sprints were likely due to oxidation of CHO due to the short duration of the exercise bout, these studies provide important evidence that aerobic metabolism remains an important contributor during HIIE, contesting the general hypothesis of negligible input.

1.2.1 Lipid Utilization and Metabolism during HIIE

As mentioned above, the dominant energy system and substrate source during exercise are affected by the duration of the acute bout, in addition to total intensity and ratio of work to rest periods^{7,33,55}. Evidence has suggested an increased importance of oxidative phosphorylation to ATP production as duration increases⁷, as well as a decrease in ability to replenish

phosphocreatine (PCr) stores with shorter resting periods. Additionally, it has been generally accepted that the increased intensity of exercise results in larger decrements of PCr⁷ and that as its resynthesis relies on availability of oxygen, aerobic capacity is inherently tied to performance during high intensities⁵⁶. Interestingly, notably higher rates of lipid oxidation have been observed in trained individuals with increased aerobic capacity compared to recreationally active individuals during an acute bout of HIIE involving six 4-minute work periods interspersed by 2 minutes of recovery on a treadmill⁵⁷.

However, while studies have been conducted to compare responses to HIIE between individuals based on training status, the specific contributions of fat oxidation remains more ambiguous, in part due to differences in work: rest ratios. While an early study reported no difference⁵⁸ in relative oxidation of CHO and lipids to energy production during interval exercise, other studies have found a marked dominance of CHO oxidation compared to lipids^{36,59,60} during HIIE/SIE, which is more widely accepted. The observed difference may be due to differences in the exercise intensities and work:rest ratios used in these studies wherein the former study used intervals at a lower intensity corresponding to 100%VO_{2max} at a 1:1 ratio to rest whereas the later studies used intervals at higher intensities (120%VO_{2peak}^{36,59,60}) with a lower work:rest ratio (1:1.5^{59,60}, 2:3³⁶). Thus, alongside an overall higher average intensity (roughly 70% VO_{2max} vs 55% VO_{2max}) the protocol employed during the latter studies correspond much more with the current definition of SIE rather than HIIE. Additionally, while the intensity of work periods during HIIE may be nearing or at 100% VO_{2max}, the average intensity of the exercise bout as a whole is lower due to the time spent at a low/rest intensity. This may tie back to the comparable rates of utilization between CHO and lipids observed in the study by Essen et al⁵⁷. Given that fat oxidation is favoured at lower exercise intensities^{7,8,22},

there could be a significant contribution of fat to energy production during HIIE/SIE. Indeed, Hetlelid et al reported notable rates of estimated fat oxidation in trained runners during an acute bout of HIIE, contributing to better performance⁵⁷. A comparison of such studies can be noted in table 1.1. However, to the best of our knowledge no one has examined IMCL utilization or activation of fat metabolism enzymes during an acute bout of HIIE.

Table 1.1. A comparison of studies examining CHO vs fat oxidation during HIIE.

	Essen et al., 1978	Christmass et al., 1999	Price et al., 2005	Price and Moss., 2007
CHO vs Fat oxidation	No difference	CHO>FAT		
Work:Rest	1:1	2:3	1:1.5	1:1.5
Work Intensity	100% VO2max	120% VO2peak		
Average Intensity	~55% VO2max	~70% VO2peak		

1.3 Sex Differences in Lipid Storage

It is well established in the literature that there are inherent differences in body composition corresponding to biological sex^{28,61–63}. At the level of the whole body, females store a greater proportion of adipose tissue than males, and in notably different areas^{28,61,63}. With respect to location, accumulation of fat mass is preferentially noted in the android and gynoid regions, respectively, for males and females^{28,61}. This has been assessed in a variety of techniques with proportions varying with the sensitivity of the tools assessed, from skinfold calipers to dual x-ray absorptiometry (DXA) scanning⁶³. Furthermore, lipid storage outside of adipose tissue also varies between the sexes^{17,27,64}. Ectopic fat, defined as storage of lipid in tissues associated with function other than adipose tissue⁶⁴, differs between the sexes with females storing more IMCL

than males^{17,27} and males tending to have greater hepatic triacylglycerol (TAG) storage^{65,66}. As the location of ectopic fat may reflect differences in metabolism and substrate preference²⁸, it then may be notable that differences in accumulation may in part support differences in fat utilization during exercise between males and females as discussed further on.

Lipid droplets residing in skeletal muscle, termed intramyocellular lipids (IMCL), are an important storage depot⁶⁷. In the neutral lipid core of the IMCL, fatty acids are largely stored as TAG⁶⁷. This is surrounded by a phospholipid monolayer, which, together with a variety of proteins, serve as the surface of the lipid droplet⁶⁷. These surface proteins are involved in the intake, storage, and release of fatty acids, termed IMCL turnover⁶⁷. While once considered a stagnant storage depot, IMCL are recognized to be an important contributor of lipids for oxidative metabolism⁶⁸. On a subcellular level, IMCL is compartmentalized to the intermyofibrillar (IMF) and subsarcolemmal (SS) regions⁴⁵. As their names suggest, SS IMCL are located between the most superficial myofibrils and the subsarcolemma, while IMF IMCL reside between myofibrils^{45,69,70}. Upon visualization using methods such as TEM, IMCL and mitochondria have been shown to interact^{17,71}, and the extent of apposition is thought to relate to its capacity to be oxidized⁷¹. As mentioned in section 1.2, the availability of FA greatly influences the extent to which lipids contribute to substrate utilization, further supporting the suggestion that IMCL may serve as an important contributor during HIIE.

Notable differences in storage characteristics have been highlighted between males and females^{13,17,19,27,45,72}. It has been reported across multiple studies that females store more IMCL than males^{13,17,19,27}. It should be noted, however, that the increase in IMCL content observed in females is due to an increase in the number, but not size, of IMCL droplets^{17,27}. Theoretically, for an equivalent volume of lipid, a greater number of smaller IMCL would have a greater exposed

surface area. An increased surface-to-volume ratio may then render the female phenotype better equipped to recruit lipolysis-mediating proteins for mobilisation of its contents^{69,73,74}. Combined with the notion that greater IMCL-mitochondria apposition is related to its capacity to be utilized⁷¹, females are again, better primed for IMCL usage through greater surface area for interaction with mitochondria.

Biological sex is also known to influence fibre type distribution¹⁹, with females and males tending toward greater proportions of type I and type II fibres, respectively¹⁹. Type I fibres notably incorporate a higher lipid content than type II fibres⁷¹, which parallels the aforementioned statement of a greater intramuscular storage of lipid in females, as IMCL. This elevated upper limit for uptake and storage of lipids⁷⁵ is not necessarily harmful, however, as type I fibres are noted as having a greater mitochondrial density and greater oxidative capacity⁷⁵⁻⁷⁷. Together with a greater surface area of IMCL, the higher mitochondrial density observed in type I fibres, of which are greater in females suggests not only an increased capacity to store lipids, but also to use them⁷⁶. These findings provide context for investigating sex differences in the distribution, localization, and storage characteristics of IMCL and how it may play into differences in IMCL specific utilization.

The sex differences in IMCL storage and metabolism mentioned in the former two paragraphs largely parallel a phenomenon referred to as the “athletes’s paradox”^{69,72,78}. A paradoxical relationship exists whereby both Type 2 Diabetics (T2D) and endurance trained athletes store a greater IMCL content than average, healthy individuals^{71,78}. Interestingly, while the increased IMCL content in T2D is correlated with insulin resistance^{69,71}, this is not the case with endurance athletes^{69,72}. Further investigation has revealed that insulin resistance is associated most with a lesser number of larger droplets in the SS region of type II muscle fibres^{69,79}. Conversely,

endurance trained athletes tend to store IMCL as a greater number of smaller lipid droplets in the IMF^{69,72,78}. Considering the shifts towards type I muscle fibres with endurance training^{80,81}, storage patterns could be interpreted as endurance athletes having a greater number of smaller IMCL, localized to the IMF region and potentially owing to a greater proportion of type I muscle fibres, while the insulin resistant and T2D is linked to fewer, larger IMCL droplets most associated with the SS region of type II muscle fibres. As priorly mentioned, females are more known to store a greater number of IMCL and have a greater proportion of type I muscle fibres, whereas males have been shown to have a lesser number of IMCL and a greater proportion of type II fibres. Interestingly, males are also shown to be at a greater risk of developing type II diabetes than females⁸²⁻⁸⁴. Further investigation to whether localization of IMCL differs between the sexes would be valuable for understanding a potential link between the susceptibility of developing type II diabetes and biological sex.

1.4 Sex Differences in Muscle Metabolism

Sex differences in fuel utilization during endurance exercise have been well established¹²⁻¹⁵, resulting in reduced fatiguability by females when matched for relative submaximal intensities¹⁵. Overall, females have a lower exercising respiratory exchange ratio (RER)^{11,12}, suggesting a greater reliance on fats during exercise than males. The greater reliance on fats during exercise is supported by a greater glycerol rate of appearance (R_a) in females than males, indicative of a greater whole body rate of lipolysis, during exercise^{12,13,15}. Despite this, there remains controversy on the source of the increased lipid reliance observed in females. No difference in plasma FFA oxidation was found between males and females at various exercise intensities [25, 45, 65 and 85% $\text{VO}_{2\text{max}}$ ^{85,86}]. However, a study by Roepstorff and colleagues¹³ did find that females oxidized 47% more plasma FFA during the last 30 minutes of a 90-minute bout of

exercise at 60% $\text{VO}_{2\text{max}}$, but this difference was not statistically significant. With respect to IMCL utilization, females have been found to oxidize more^{13,19,87}, similar amounts^{17,18}, or less¹⁶ IMCL than males during endurance exercise. Differences in the findings of these studies with respect to sex-based differences in IMCL utilization during exercise may be due to methodological limitations as detailed in the following paragraph.

Discordance in the findings of the influence of sex on IMCL utilization during exercise may be due to differences in methodology used to determine IMCL utilization. Methods used to determine IMCL content in the aforementioned studies include the biochemical extraction method^{88–90}, transmission electron microscopy (TEM)^{17,91} and proton magnetic resonance spectroscopy (H-MRS)^{10,16,18,92,93}. With respect to sensitivity in testing, evidence shows that the biochemical method is accompanied by the shortfall of contamination with extramyocellular lipids (EMCL)^{17,94}. Moreover, contamination by EMCL may not only affect reporting of IMCL usage over exercise but of observed differences between biological sexes^{88–90}. Notably, studies using the biochemical extraction method have reported IMCL utilization only in females, with no change in males^{13,87}. However, while this supports the notion that lipids are preferentially used by females as a fuel source^{11,17,27,95}, the polarity of these results have been disputed in that there is a low likelihood that males do not use IMCL at all over an acute bout of exercise^{13,19,87}, especially given that increases in IMCL stores are noted in endurance athletes of both sexes^{69,72,95}. Additionally, in one study where it was reported that males did not use IMCL during exercise, measured substrate oxidation accounted for 99% of leg oxygen uptake in females, but only 72% of leg oxygen uptake in males¹³. The authors suggested that these findings indicate that males relied on other sources of lipids such as very low-density lipoprotein-TAG or extramyocellular lipids; however, this may have been IMCL that were used but missed due to the

limitations of the biochemical extraction method. Furthermore, despite a large agreement in TEM and H-MRS results²¹, differences may arise from a lack of standards in place for transmission electron microscopy analysis. While TEM allows for the visualization of subcellular compartments, a noted shortcoming is the analysis of few muscle fibres per each sample^{96,97}, thus building a profile that may not be representative of the whole muscle. While H-MRS provides an ideal alternative to both electron microscopy and biochemical methods, and is widely considered a gold standard method, it is comparatively expensive and may not be feasible to employ²¹. Thus, if using TEM analysis to determine IMCL content and utilization it is important to obtain images from multiple muscle fibres. When doing so the correlation between TEM and H-MRS is strong ($r=0.921$)²¹. Lastly, differences observed between studies may also be due to improper matching of male and female participants. As males and females differ in body composition it is imperative that they are matched for fitness based on VO_2max relative to lean mass, rather than relative to body weight⁹⁸. In the one study that reported that males used more IMCL than females¹⁶, males and females were not matched appropriately and males were more trained than females, which likely explains the greater IMCL usage. Thus, trials investigating sex-based differences in IMCL utilization need to ensure they use appropriate methods to match for participants cardiorespiratory fitness. To date no one has examined whether there are sex differences in IMCL utilization during an acute bout of HIIE.

1.4.1 Sex differences in lipid metabolism protein content and enzyme activity

Sex differences in lipid metabolism do not appear to be restricted to one singular mechanism²⁸. Instead, females have elevated expression, content, and/or activity of enzymes/proteins involved at multiple steps, from the freeing of FA from TAG storage in adipose stores, transport into the muscle, turnover of IMCL stores, as well as transport into the

mitochondria to be utilized^{28,87,99,100}. To start, Figure 1.2 shows an overview by which TAG stores are broken down in a step-wise fashion to diacylglycerol (DAG), monoacylglycerol (MAG) by adipose triglyceride lipase (ATGL), and hormone sensitive lipase (HSL) respectively, and eventually freeing the last FA from the glycerol backbone¹⁰¹. The complete breakdown of TAG to 3 free fatty acids and a glycerol backbone may not always occur, however, the resultant fatty acids may then go into circulation for transport into active tissue such as skeletal muscle⁷⁶.

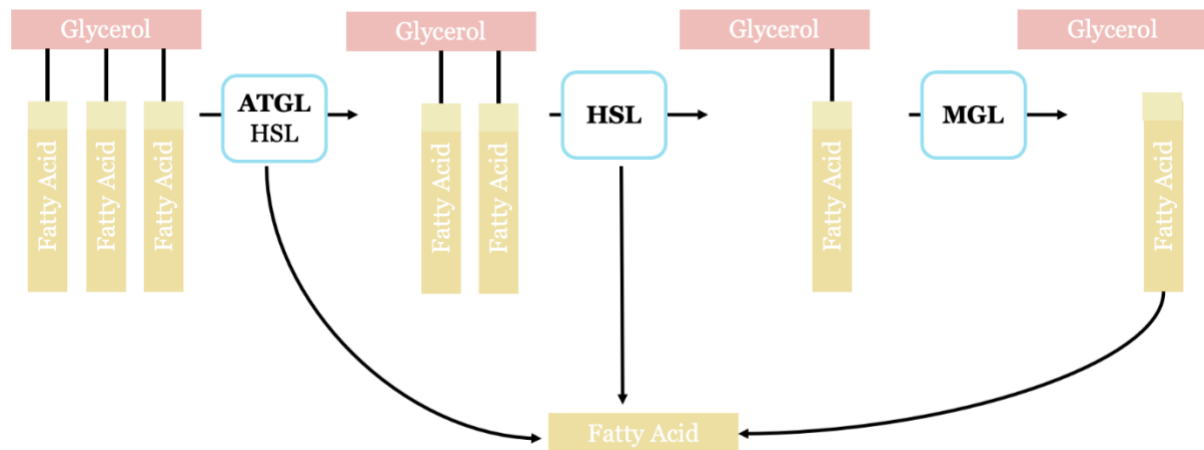


Figure 1.2. Schematic for the breakdown of TAG, involving ATGL, HSL and MGL. Adapted from Hofer et al. (2020)¹⁰¹. Bold denotes the main enzyme responsible during each stage of breakdown.

Past this point, fatty acids must enter skeletal muscle. This occurs by passive diffusion or utilizing transport proteins such as fatty acid transporter (FAT)/CD36, Fatty acid transport protein (FATP) and/or fatty acid binding protein (FABP)¹⁰². Acetyl-CoA synthetase will then act, forming a FA-CoA¹⁰³, which may then be fated towards utilization by the mitochondria, or storage within skeletal muscle as IMCL^{78,103}. If fated for storage, mitochondrial glycerol-3-phosphate acyltransferase (GPAT) facilitates TAG synthesis through the addition of a glycerol backbone to 3 FA¹⁰⁴. Furthermore, members of the perilipin family of proteins are involved in

IMCL turnover through influence over TAG synthesis, breakdown, and IMCL-mitochondria contact^{68,103,105}. FA destined to be used as a fuel source must enter the mitochondria by way of the “carnitine shuttle” consisting of CPT-1, translocase, and CPT-2^{45,78}, thereafter undergoing beta oxidation²⁸. A depiction of these processes, and sex differences may be noted in Figure 1.3.

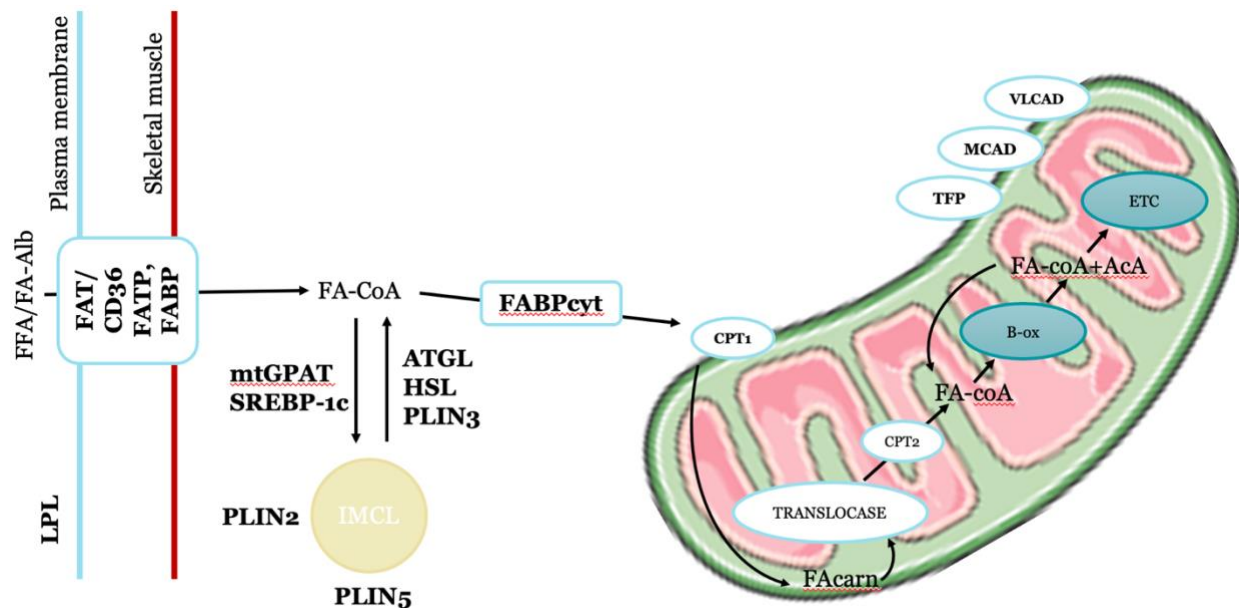


Figure 1.3 Depiction of lipid metabolism and IMCL turnover pathways, adapted from Beaudry and Devries (2018)²⁸. Enzymes/proteins that are bolded have been found to be greater in females than males at the level of mRNA expression, protein content and/or enzyme activity.

Studies examining sex differences in metabolism have reported that females may be advantaged towards lipid oxidation at multiple steps including FA transport into the muscle, storage, and utilization²⁸. Not only do females show greater expression and content of proteins/enzymes involved in lipid oxidation, including trifunctional protein alpha (TFP-alpha), and CPT1¹⁰⁶, they also have greater content of beta oxidation enzymes including medium chain acyl-CoA dehydrogenase (MCAD), and very long chain acyl-CoA dehydrogenase (VLCAD)¹⁰⁷. Females have greater content of both PLIN2⁶⁸, which supports TAG synthesis and opposes IMCL lipolysis, and GPAT¹⁰⁶. Together, this suggests an increased ability for lipid droplet formation in

females. This difference does not end at IMCL synthesis but rather extends to proteins involved in IMCL utilization²⁸. Again, key players involved in TAG mobilisation such as adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and perilipin-3 (PLIN3) are documented to be influenced by sex^{87,108}. Interestingly, while females have elevated content of ATGL, HSL and PLIN3^{87,108}, it was noted that there was no difference in HSL activity⁸⁷. Past the liberation of TAG from IMCL, the resultant FAs enter the mitochondria for oxidation.

Lastly it is hypothesized that the availability of FA for oxidation influences the extent to which it contributes to energy production⁸, and thus it is thought that the utilization of FA released from IMCL may be optimized by IMCL-mitochondria apposition^{20,39,82,88}. It has been observed that endurance training elicits an increase in IMCL-mitochondria co-localization regardless of sex^{27,79,91}, however this increased apposition is only found in females following an acute bout of exercise¹⁷. The protein perilipin-5 (PLIN5), is responsible for IMCL-mitochondria apposition^{68,103}. Interestingly, females have greater protein content of PLIN5 as compared to males⁶⁸. Together, these results suggest that females have a greater capacity for IMCL turnover, and oxidation when compared to males. However, because investigations have largely employed endurance exercise protocols, whether this sex difference persists for exercise at higher intensities remains unclear.

1.5 Sex Differences are mediated by Estrogen

Given the differences observed in substrate utilization during exercise, the mechanisms by which sex differences occur are a logical course of investigation. It has been posited that sex hormones, specifically estrogen, are responsible for mediating these differences^{106,109}. Metabolically, oestrogen targets adipose tissue, skeletal muscle and the liver to increase lipolysis^{110,91,92,100}. Many trials have been conducted to investigate the metabolic effects of

estrogen using both rodents and humans. Rodent trials have used supplementation to either male rats or ovariectomized females^{111–116}. These studies have reported that estradiol increases fat oxidation and decreases utilization of hepatic glycogen during exercise^{112–116}. In humans, common methods of examining the effects of estrogen have been to administer 17- β estradiol (E2) to males^{98,117–119} or amenorrheic females¹²⁰ or to observe differences in metabolism over the course of the menstrual cycle¹⁰⁶. While some studies in males and amenorrheic females have shown no effect of E2 supplementation on RER^{117,119}, others in males have reported decreases^{118,121}. Furthermore, these studies also report a decrease in CHO oxidation^{118,121} and an increase in lipid oxidation^{121,122}. Specifically, administration of E2 to males and amenorrheic females has been consistently found to decrease glucose Ra, indicative of a reduced reliance on hepatic CHO during exercise^{117,118,120}. However, despite an effect on glucose Ra and rate of disappearance (Rd), glycerol Ra, a measure of whole-body lipolysis, did not change with E2 supplementation^{117,120}. Differential findings are likely related to differences in dose and length of treatment, with doses meant to mimic estrogen concentrations found in the luteal phase of the menstrual cycle administered for 8 days appearing to have the greatest effects. Importantly, when E2 is administered to males it not only increases estrogen concentration, but decreases testosterone concentration as well. As such it cannot be determined from these trials whether the effects are the result of increased estrogen or decreased testosterone. However, Braun and colleagues¹²² found no differences in fuel metabolism when males were tested at low (induced by pharmacological suppression of testosterone), high (induced by transdermal testosterone patches) or physiological (no intervention) testosterone levels. Together, the findings of these studies support a role for estrogen in mediating fuel utilization during exercise.

Definitive results are lacking on the effect of menstrual cycle phase on substrate utilization in humans^{115–119}. However, a number of studies have reported elevated FFA oxidation when tested in the luteal phase as compared to the follicular phase^{116,118,119}. Estrogen rises from the mid-follicular phase to a peak at the end of the follicular phase, drops and then steadily rises to another secondary peak during the luteal phase¹²⁰. Another study comparing eumenorrheic women at the mid-follicular versus mid-luteal phases saw participants cycling at 60% $\text{VO}_{2\text{max}}$ for 90 minutes, followed by a depletion protocol of cycling at 100% $\text{VO}_{2\text{max}}$ for 4x1 minute, and after 72 hours of rest, cycling at 70% $\text{VO}_{2\text{max}}$ until exhaustion¹²². The study reported a nonsignificant trend towards prolonged time to exhaustion in the group tested in the mid-luteal phase¹²². A small sample size may contribute to the nonsignificant result, as this study tested only 6 individuals¹²². The discordance observed in human studies may in part be due to differences in testing in the early-mid follicular, mid-late follicular, ovulation, or luteal phases, small sample sizes, dosage and duration of supplementation, and duration and intensity of exercise.

Notable mechanisms by which estrogen may influence substrate utilization is through affecting catecholamines (epinephrine and norepinephrine)¹²³ and the expression and content of key proteins/enzymes involved in lipid metabolism^{87,108,111,115,116,124}. Epinephrine and norepinephrine both increase from rest to exercise¹²⁵ in an intensity-dependent manner^{126,122,124–126}. Epinephrine has been reported to affect fatty acid availability by stimulating lipolysis^{46,125–127}. During exercise catecholamines increase to a greater extent in males than females^{19,128–130}, which given that catecholamines increase lipolysis is opposite to what would be expected given that lipolysis and fat oxidation are greater in females than males during exercise. However, while catecholamines increase to a greater extent in males than females, females have a greater

sensitivity to the lipolytic effects of catecholamines¹³¹. Specifically, females have greater β -adrenergic (which increases lipolysis when stimulated) and lower α -adrenergic (which decreases lipolysis when stimulated) sensitivity^{132,133}, which results in a greater rate of lipolysis and fat oxidation during exercise in females compared with males.

Additionally, it has been posited that estrogen may influence the expression, content, and activity of proteins/enzymes involved in fat oxidation. Indeed, at rest, studies have shown greater expression and content of CD36¹³⁴, and HSL⁸⁷, and greater mRNA expression of lipoprotein lipase (LPL)¹³⁴, FATP1¹³⁵, FABP_{PM}¹³⁴, CPT1⁴⁷, mtGPAT⁹⁸, BHAD¹³⁶ and TFP¹³⁶ in females. While expression and content of proteins/enzymes do not indicate activity, these findings suggest greater capacity for FA entry into the cell, TAG synthesis, TAG mobilisation, and lipid oxidation in females. Moreover, studies supplementing with E2 have reported increases in mRNA expression and protein content of key proteins/enzymes¹⁰⁶. A study by Maher et al¹²⁴ found that 8 days of supplementation with 17 β -estradiol to males resulted in increased mRNA expression of PGC-1 α , and protein content of MCAD. As priorly mentioned, MCAD is a protein that participates in beta-oxidation of medium chain fatty acids¹⁰⁷. Further, it has been found that increased PGC-1 α expression may influence beta oxidation through components within the electron transport chain^{137–139}. This includes the increased expression of cytochrome c, cytochrome C oxidase (COXIV), and the beta subunit of ATP synthase^{138,139}. Furthermore, Fu and colleagues found that menstrual cycle, estrogen supplementation in men, and as an even more substantial predictor, sex, influenced the mRNA expression of multiple genes associated with lipid metabolism¹⁰⁶. Collectively these findings support a role for estrogen in mediating lipid storage and metabolism^{28,107,136}.

Chapter 2: Rationale, Purpose, Objectives and Hypotheses

2.0 Rationale

There are well known sex-based differences in both fuel storage and metabolism during exercise¹⁻⁸. With respect to lipid storage, females are known to store greater amounts of adipose tissue than males, as well as in notably different regions of the body⁹⁻¹¹. Moreover, females also store a greater amount of lipid in skeletal muscle as lipid droplets termed intramyocellular lipids (IMCL). Importantly, females store this greater amount of lipid as a greater number of smaller droplets, which is a storage pattern that may support its usage more during exercise¹²⁻¹⁵. Sex based differences in metabolism during exercise are classically showcased by females relying to a greater extent on lipids as a fuel source during MICE^{12,14}. This has been evidenced by a number of methods such as a lower exercising RER^{2,16}, as well as a greater glycerol rate of appearance^{1,2,12}. However, despite females relying on lipids to a greater extent during exercise, as well as muscle storage patterns suggesting females are primed for utilizing lipid fuel sources, whether females use IMCL to a greater extent during exercise is controversial. Conflicting publications have reported males using IMCL to a lesser^{12,13}, equal^{14,17}, or greater extent¹⁸ than females. This may be due to preferential usage of subcellular compartments, which has not been explored, as well as not properly matching males and females for fitness and limitations associated with methods to determine IMCL content. Due to the nature of HIIE substantially less investigation has been conducted to examine the contribution of lipids towards energy demands. To our knowledge, IMCL utilization, especially noting sex differences in subcellular utilization, have not been examined during an acute bout of HIIE.

2.1 Purpose

The purpose of this study was to determine whether IMCL are used during an acute bout of HIIE and whether sex influences the extent of utilization or morphological characteristics.

2.2 Objectives

- 1) To determine whether IMCL are utilized during an acute bout of HIIE
- 2) To determine whether a sex difference exists in IMCL utilization during an acute bout of HIIE
- 3) To characterize location-specific (SS, peripheral IMF, central IMF) IMCL utilization during HIIE
- 4) To determine whether a sex difference exists in location-specific IMCL utilization
- 5) To determine whether sex influences IMCL storage characteristics and localization
- 6) To determine whether sex influences RER and whole body substrate metabolism during an acute bout of HIIE
- 7) To determine whether there are sex-based differences in lipid metabolism (PLIN2, PLIN3, PLIN5, ATGL, CPT1, β HAD content) and aerobic metabolism (CS, COXI, COXII, COXIII, and COXIV content).

2.3 Hypotheses

- 1) IMCL will contribute to energy production during HIIE, evidenced by a decrease in IMCL area density, and an increase in IMCL-mitochondrial proximity post-exercise.
- 2) IMCL utilization will be greater in females than males
- 3) There will be greater reliance on IMCL in the peripheral intermyofibrillar region, followed by the subsarcolemmal region, and the central intermyofibrillar regions, respectively.
- 4) Females will use more lipid in the peripheral IMF, SS and central IMF regions than males; however, there will be no differences in the relative contribution of IMCL from each site to total IMCL utilization between males and females

- 5) Females will have a greater number of similar sized IMCL, with more localized to the IMF region than males
- 6) RER and CHO oxidation will be lower and fat oxidation will be higher in females compared with males during HIIE.
- 7) Females will have a higher content of lipid and aerobic metabolism enzymes than males.

Chapter 3: Methods

3.0 Participants

This study has been reviewed and approved by the University of Waterloo Research Ethics Committee (ORE# 22477). Twenty-four participants equally divided between males (n=12) and females (n=12) were recruited for participation in this study. The targeted recruitment group was young, healthy and recreationally active individuals. Prospective participants who fell between the ages of 18-30 years had their eligibility evaluated using the following inclusion and exclusion criteria. To satisfy the stipulation that our participants were healthy and only recreationally active, prospects were excluded from either end of the spectrum of training status and body composition. Thus, individuals were excluded if BMI was $>27\text{kg/m}^2$, if their completed Get Active Questionnaire recommend refraining from exercise, or if they were unable to safely perform an acute exercise bout. Conversely, female and male prospects were excluded if they achieved relative $\text{VO}_{2\text{peak}}$ values greater than 44 or 51 $\text{mL}\cdot\text{kg}^{-1}\cdot\text{m}^{-1}$, respectively, or habitually participated in more than two resistance training, or three cardiovascular training sessions per week. To account for training status, males and females who satisfied all requirements to be included were matched by maximal capacity for oxygen consumption ($\text{VO}_{2\text{peak}}$) relative to fat free mass. Participants were excluded if diagnosed with any chronic health condition prior to this study, including but not restricted to cardiorespiratory, digestive, or metabolic disorders. Furthermore, potential participants were excluded if they were taking anti-platelet or anticoagulant medications, had undergone imaging using contrast agent infusion or a barium swallow within 3 weeks prior to the trial, or had any allergies to local anaesthetics. Standardized mid-follicular phase testing windows were employed for females to control for the effect of the menstrual phase on muscle metabolism during exercise. Thus, all sessions completed by female

participants fell within days 4 and 9 of their menstrual cycle, confirmed by the onset of menses. Moreover, prospective female participants were excluded from the trial for both suspected or confirmed pregnancy, breastfeeding, or for the use of certain hormonal contraceptives including a hormonal IUD or monophasic oral pills. The risks, benefits, procedures and protocols were detailed for all participants both verbally and as a written outline. Written consent was collected prior to the subjects' continuation with the trial.

3.1 Study Design

3.1.1 Study Overview

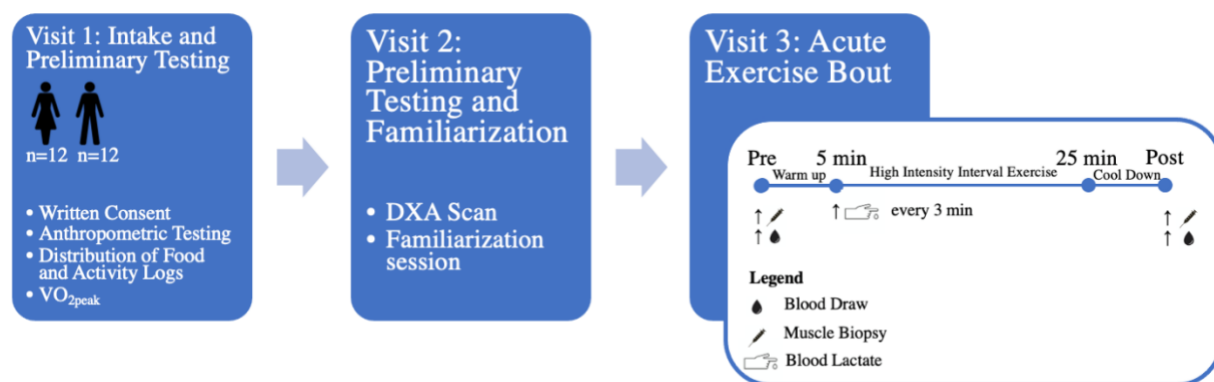


Figure 3.1: A General Outline of Study Schematic

The research conducted in this thesis was part of a larger trial examining whether sex influenced insulin sensitivity, pancreatic beta-cell function and muscle metabolism during different modes of exercise. Previous publications from this cohort include an examination of whether sex influenced post-exercise insulin sensitivity following an acute bout of HIIE¹⁴⁰ an examination of whether sex and/or exercise mode influenced the effects of acute exercise on pancreatic beta-cell function¹⁴¹ and whether exercise mode influenced vascular dynamics during a post-exercise glucose challenge¹⁴². For this study participants were asked to visit the laboratory a

total of three times. Preliminary testing occurred on the first two visits, with the acute exercise bout being performed on the third. A detailed overview of the acute HIIE bout can be found in 3.1.2.

At the first visit, a member of the research team provided a verbal explanation of the trial procedures. At this time, participants received a detailed overview of the study outline, and had an opportunity to address any questions or concerns before providing written informed consent of their participation. Participants were then asked to complete both the Get Active Questionnaire (GAQ) and a general health screening questionnaire prepared by the research team. Individuals also had general anthropometric measures including height and weight taken. If the participant met general anthropometric requirements (BMI) and there were no contraindications to exercise, as indicated by the two aforementioned questionnaires, they proceeded to a graded exercise test (GXT). A ramp protocol was employed for the GXT, which was performed on a cycle ergometer (Ergoline GmbH, Bitz, Germany). A metabolic cart (Vmax, Vyair Medical, Mettawa, IL, USA) was used to assess their $\text{VO}_{2\text{peak}}$ associated with the test. An initial two minute warm-up at an intensity of 50 Watts preceded the graded exercise phase. Thereafter, the intensity increased at a rate of 1 Watt every 2 seconds. During the entirety of both phases, participants were required to achieve and sustain a cadence of at least 60 revolutions•minute⁻¹. Requirements of test termination included when participants failed to sustain the 60 revolutions•minute⁻¹ pace, or upon volitional failure. Starting at warm up, heart rate was monitored using a Polar Heart Rate Monitor (Polar, Lachine, QC, CA), blood lactate was measured using lactate sensor strips (EKF Diagnostics, Cardiff, UK) and a Lactate Scout Plus (EKF, Penarth, England) at intervals of thirty seconds and one minute, respectively. Additionally, participants were asked to give a rating of their perceived exertion (RPE) at one-

minute intervals throughout the test using the 6-20 Borg Scale. The individual's $\text{VO}_{2\text{peak}}$ was determined as highest oxygen consumption volume corresponding to an averaged 30-second interval on the metabolic cart. After the test, participants were provided with a 7-day physical activity log, a 3-day food tracking log, and a PiexoX pedometer (StepsCount, Deep River, ON, Canada). The diet and activity data was used for assessment of habitual dietary intake and physical activity in both sexes.

The second laboratory visit consisted of a body composition assessment and a familiarization to the HIIE protocol. Participants underwent a dual x-ray absorptiometry (DXA) scan on the Hologic Discorvery W (Hologic, Mississauga, ON, Canada). After the scan was completed, the technician used the accompanying QDR APEX software (Version 4.5.3 Hologic, Mississauga, ON, Canada) to determine the participant's body composition. Prior to the participants arrival, the intensity required to evoke a heart rate corresponding to 90% of the maximal heart rate (HR_{max}) achieved during the $\text{VO}_{2\text{peak}}$ test was calculated. The familiarization protocol mimicked the high intensity interval protocol completed at visit 3. A five-minute warm up at 50 Watts started the familiarization protocol. Thereafter, participants cycled for 60 second intervals, alternating between the wattage corresponding to 90% HR_{max} , and 50 Watts. These values corresponded to periods of high and low intensity, respectively. These intervals were repeated three to six times in order to determine if the calculated wattage was capable of eliciting the target heart rate. If upon the third interval, participants reached the target heart rate, they proceeded to a five-minute cool-down under the same conditions as the warm-up. If at the third interval the participant was not within range of the target heart rate, the intensity was altered in order to achieve the desired heart rate response. Failure to reach the target heart rate range in the next three intervals resulted in cessation of the session. Calculations were repeated and

familiarization was repeated on another day to confirm appropriate wattage to elicit the conditions of the acute HIIE bout that followed.

The acute HIIE bout occurred on the participants' third visit to the laboratory. Prior to this visit, individuals completed a 12-hour fast, and abstained from exercise at a moderate to vigorous intensity for 72 hours.. Upon arrival at the laboratory, participants had a muscle biopsy collected from the *vastus lateralis* muscle. The exercise bout commenced immediately after. The HIIE protocol and sample collection schematic is detailed below in Figure 2. After an initial five-minute warm up set at 50 W, participants performed 10 rounds of intervals, followed by a five-minute cool down also set to 50 Watts. Each interval lasted 60 seconds, alternating between 50 Watts, and a workrate value determined to elicit 90% of their HR_{max} , as determined by their VO_{2peak} and familiarization sessions. Starting at the fifth minute, blood lactate was assessed every three minutes via finger prick, and heart rate and rating of perceived exertion every minute. Indirect calorimetry data was collected using breath by breath analysis and averaged across thirty second intervals on the metabolic cart. Intervals of breath data collection were during warm-up (minutes 0-5), midway through the exercise intervals (minutes 12-17), and cooldown (minutes 25-30). These values were used to calculate whole-body substrate utilization. Immediately after the exercise bout, participants dismounted the cycle ergometer and

immediately another muscle biopsy was collected from the priorly mentioned site.

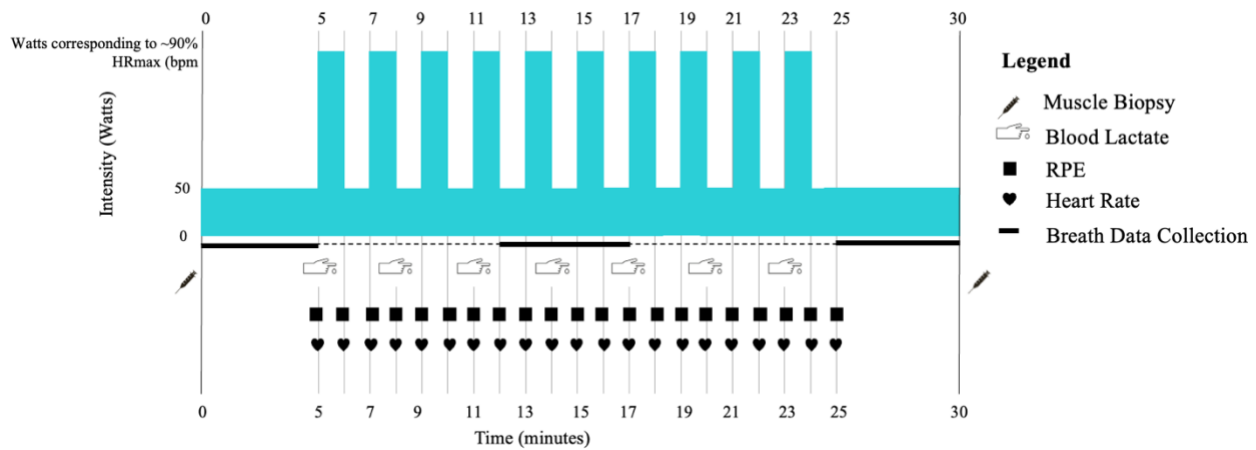


Figure 3.2: HIIE Protocol and Collection Schematic

3.1.2 Sample Collection

Muscle biopsy collection occurred prior to, and immediately following the HIIE bout. Using a Bergstrom needle under suction, the muscle biopsy was taken from the *vastus lateralis* muscle as previously described by Beaudry and colleagues in 2023¹⁴⁰. To allow for immediate sampling post exercise, two incisions ~5cm apart were made prior to exercise with one being accessed for the pre exercise biopsy and the other covered with a sterile tegaderm and used for the post-exercise biopsy. Macroscopic processing to remove fat and connective tissue was performed and the sample was divided with one piece being placed into 2% glutaraldehyde and stored at 4°C prior to further processing for TEM analysis. The other piece was sectioned and snap frozen in liquid nitrogen and stored at -80°C until subsequent analysis western blotting. Blood lactate was assessed from blood obtained via finger prick using lactate sensor strips (EKF Diagnostics, Cardiff, UK) and a Lactate Scout Plus (EKF, Penarth, England).

3.2 Analysis

3.2.1 Dietary Intake Analysis

Data from each participants 3-day diet log was entered into the online ASA24 dietary assessment tool (Version Canada (2018), National Cancer Institute, Bethesda, MD). Data was exported as a record per day, per each respondent, for food group and nutrient values totaled from all beverages, supplements, and foods consumed. These values were then used to assess the daily caloric intake (kcal/day) in addition to the absolute intake of the macronutrients. Fat intake was also assessed for each individual's consumption of monounsaturated fatty acids, polyunsaturated fatty acids, and saturated fatty acids. These values were then expressed as a percentage contribution toward the daily caloric intake, and were also expressed as intake relative to body mass, FFM, and lower body fat free mass (LB FFM) for comparisons between the sexes.

3.2.2 Indirect Calorimetry

3.2.2.1 Determination of TEE during the exercise bout, and absolute rates of CHO and Fat oxidation

Mean oxygen consumption (VO_2 , L/min) reported by a gas exchange measurement system (Vmax Encore CPET Systems, Germany) during each interval of exercise, warm up, high intensity intervals, low intensity intervals, and cool down were taken and multiplied by the duration of each exercise interval in minutes for each period. This value was then multiplied by 5 to estimate the total energy expenditure (kcal) per each period measured.

3.2.2.2 %CHO and %Fat Contributions

The validated equations below were generated by Elia and Livesey¹⁴³, and assume negligible contribution of protein as a fuel source^{143,144}. %CHO and %fat oxidation were approximated by substituting the RER reading from the metabolic cart as RQ.

$$\%CHO = \frac{[(5.045 \times RQ) - 3.582]}{[(0.36 \times RQ) + 1.103]}$$

$$\%Fat = 1 - \%CHO$$

As previously mentioned, RER averages were calculated for the warm up, high intensity intervals, low intensity intervals, and cooldown. These were then used in the equation to estimate %CHO and %Fat oxidation. It should be noted that while we collected breath data from minutes 12-17, thus consisting of two high intensity intervals and three low intensity intervals, for consistency, data from only two of the low intensity intervals were used in the calculations. The estimation of CHO and fat oxidation was determined by multiplying the %CHO and %Fat by the duration of each respective time period, and dividing by 4 or 9 for CHO and fats, respectively. The rate of oxidation was then obtained by dividing this value by the duration of each respective time period again.

3.2.3 Western Blot Analysis

Samples intended for western blot analysis were stored in a cryovial and submerged in liquid nitrogen immediately upon removal from the *vastus lateralis* and processing. Samples were then be transferred and stored in a -80° freezer until further analysis. Ice cold 25mM Tris buffer was prepared [175mg Tris-HCl, 16.75mg Trizma Base, 25mL ddH₂O] at pH 7.2. This was then used in the preparation of ice cold homogenization buffer, also at pH 7.2 [25mM Tris based buffer, 0.5% (v/v) Triton X-100, PhosSTOP phosphatase inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany), cComplete Mini, EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany)]. Muscle specimens were weighed and transferred into Biopur Safe-lock Eppendorf tubes (Eppendorf Canada Ltd., Mississauga, ON,

Canada) with ice-cold homogenization buffer, and a stainless steel bead (Qiagen, Toronto, ON, Canada). The ratio of homogenization buffer to muscle was 10 μ L per every 1mg tissue. Eppendorfs were be loaded onto a pre-chilled rack and homogenized using the TissueLyser II (Qiagen, Toronto, ON, Canada), at a frequency of 20 cycles $\cdot$ second⁻¹. The duration of homogenization was 40 seconds. Subsequently, homogenized samples were loaded onto the Eppendorf Centrifuge 5804 R (Eppendorf Canada Ltd., Mississauga, ON, Canada) set to 4°C and centrifuged for 10 minutes at 10 000G. Upon separation, the pellet was stored at -80°C for additional assays. The supernatant was used during a bicinchoninic acid (BCA) assay for protein quantification. ‘Western ready’ samples were prepared with supernatant, ddH₂O and Laemmli Buffer (0.5M Tris-HCl, ddH₂O, 10%SDS, 1% bromophenol blue, glycerol, and β -mercaptoethanol), with a sample concentration of 2 μ g $\cdot$ μ L⁻¹.

Detection of proteins of interest was performed using SDS-PAGE and Western Blot techniques. Five microlitres of sample, corresponding to 10 μ g of protein, was loaded along with a standard curve of 5, 10, 15, and 20 μ g of pooled sample, and a Kaleidoscope Protein Ladder (Precision Plus Protein Standard, BioRad, Hercules, CA, USA) on a 15% Criterion TGX Stain Free gel (BioRad, Hercules, CA, USA). Gel electrophoresis was run at 200 volts for 45 minutes, with subsequent protein transfer onto an Immun-Blot PVDF membrane (BioRad, Hercules, CA, USA) using the Trans-Blot Turbo Transfer System (BioRad, Hercules, CA, USA). The Chemidoc MP (BioRad, Hercules, CA, USA) was used for confirmation of the protein transfer, as well as protein detection. Upon confirmation of protein transfer, membranes were blocked in 5% skim milk in 1X Tris-buffered saline and tween-20 (1X TBS-T) (see table 1 for details) for two hours. Subsequently, membranes were incubated with primary antibody suspended in 0.5% or 5% skim

milk in 1X TBS-T overnight at 4°C (details included in table 1). Three 5-minute washes in 1X TBS-T followed, to prevent binding of secondary antibody to excess primary. Following the three washing cycles, the membranes were incubated in the corresponding secondary antibodies (see table 1 for details) at room temperature for an hour. Again, excess antibody was removed through three 5-minute washes in 1X TBS-T. To detect the protein of interest, the membranes were incubated in Clarity ECL Western Blotting Substrate (Biorad, Hercules, Ca, USA) or Clarity Max ECL Western Blotting Substrate for two minutes prior to chemiluminescent and colorimetric imaging on the Chemidoc MP imaging system.

In the event that the membrane was being probed for more than one protein, the following stripping and reprobing protocol occurred. To remove residual Clarity ECL Western Blotting substrate, membranes underwent three 5-minute washes in 1X TBS-T. Antibodies were stripped off with two 15-minute washes in mild stripping buffer (SDS, Tween-20, Glycine, ddH₂O, pH 2.2), and two 10-minute washes with 1X PBS (NaCl, Na₂H₂PO₄, Na₂HPO₄•7H₂O, ddH₂O, pH 7.2). Following two 5-minute washes in 1X TBST, membranes were incubated for 15 minutes in secondary antibody and Clarity ECL Western Blotting substrate for 1 minute, then imaged using the Chemidoc MP imaging system for confirmation of stripping. The membranes then underwent another three 5-minute washes in 1X TBS-T to remove the secondary antibody and Clarity ECL Western Blotting Substrate. Appropriate blocking and reprobing with the next protein's corresponding antibody followed.

Quantification and analysis of both total protein content from the stain free blots, and relative protein content using bands from chemiluminescent imaging, was done on the ImageLab (Version 6.0.1, BioRad, Hercules, USA). Normalization using the standard curve of pooled

sample was also done using ImageLab. A complete list of antibodies and corresponding treatment can be found in Table 1.

Table 3.1: Proteins of interest, and corresponding antibodies, dilutions, and blocking agents intended for use with Western Blot techniques.

Protein	Antibody	Blocking Agent	1° Antibody Dilution	2° Antibody Dilution
Adipose triglyceride lipase (ATGL)	Cayman Chemical 10006409	5% skim milk in 1X TBS-T	1:200	1:10000
Carnitine palmitoyltransferase (CPT1)	Abcam 128568		1:1000	
Short chain 3 hydroxyacyl-CoA dehydrogenase (SCHAD)	Abcam 154088			
Citrate synthase (CS)	Abcam 96600		1:3000	
COX (I-IV) cocktail	Abcam 110411		1:1000	
PLIN2	Progen 610102			
PLIN3	Abcam 47638	0.5% skim milk in 1X TBS-T		
PLIN5	Progen GP31S	5% skim milk in 1X TBS-T		

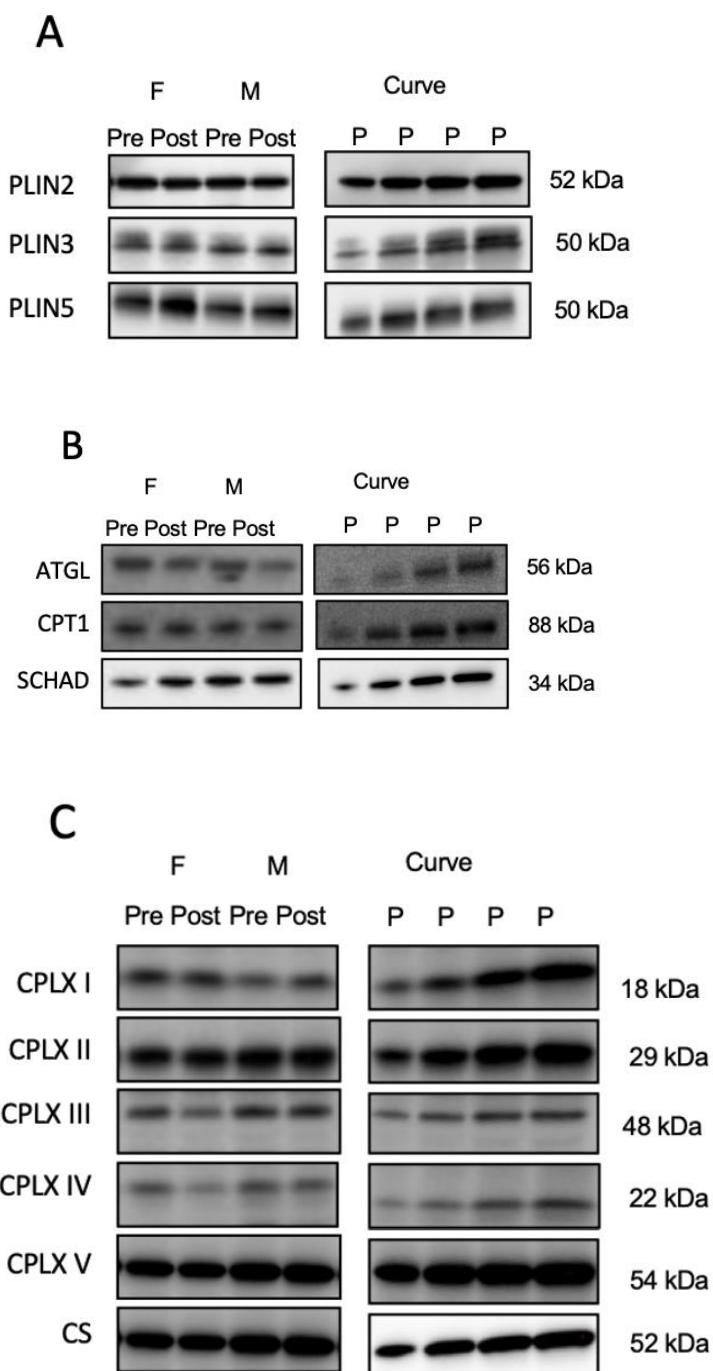


Figure 3.5. Representative images of Western blots. A) Protein content of PLIN2, PLIN3, and PLIN5. B) ATGL, CPT1, and SCHAD and C) Complex I, Complex II, Complex III, Complex IV, Complex V, and CS. Samples from prior to and after the acute HIIE exercise bout were run on each gel alongside a standard curve from a pool of all samples (P).

3.2.4 Transmission Electron Microscopy Analyses

Upon removal from the *vastus lateralis*, part of the muscle biopsy sample was sectioned off for transmission electron microscopy (TEM) analysis. This sample was fixed in 2% glutaraldehyde. The fixed samples were then stored at 4°C until the embedding process. Technicians at McMaster University's Electron Microscopy Facility completed the embedding process. During this, specimens were fixed in 1% osmium tetroxide and dehydrated in ethanol. Fixed and dehydrated samples were then embedded in Spurr's resin. Further processing and TEM analysis will occur at the University of Waterloo.

The Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems, Concord, CA, USA) was used for preparation of the samples prior to TEM analysis. First, samples were isolated within the resin, and exposed using an uncoated 0.009-inch, single edged, stainless steel blade with aluminum backing (GEM, AccuTec Inc., Verona, VA, USA). Longitudinal slicing of the samples occurred using an ultra 45° diamond knife (DiAtome, Hatfield, PA, USA) on the ultramicrotome. Samples were cut to 70nm and smoothed by exposure to chloroform vapors. Smoothed samples were then transferred to Copper 200 mesh grids (Electron Microscopy Sciences, Hatfield, PA, USA) using a perfect loop (Electron Microscopy Sciences, Hatfield, PA, USA). Samples were stained using the Uranyl Acetate and Lead Citrate double staining method¹⁴⁵. Samples were incubated in a saturated uranyl acetate (Polysciences Inc, Warrington, PA, USA) solution for five minutes, and a 0.4% lead citrate (Polysciences Inc., Warrington, PA, USA) solution for one minute, interspersed by 1-minute washes in 50% EtOH and double distilled water, respectively¹⁴⁵. Double distilled water was boiled, then cooled while covered to ensure that all solutions made were free of carbon dioxide¹⁴⁵. After the final wash in CO₂-free double distilled water, grids were dried and stored overnight before transmission electron microscopy imaging¹⁴⁵.

Stained samples were visualised on the Phillips CM10 transmission electron microscope (Phillips Electronics, Eindhoven, The Netherlands). Image capture was performed at a magnification of 7900x. For each sample, a total of 20 images from at least 10 different fibres were taken. The breakdown of image distribution was as follows: 10 of the subsarcolemmal region, 3 including a nucleus and 7 without, and 10 of the intermyofibrillar region. A total of 900 images were taken for this study. Image analysis proceeded using IMAGE J software (Version 1.51, National Institute of Health, USA). Magnification and scale references were input into the application to allow for analysis of structures according to actual size. IMCL sphericity, IMCL diameter, IMCL ferret(nm), IMCL perimeter(nm), IMCL area (μm^2), IMCL perimeter to area ratio, IMCL aspect ratio, IMCL number/area ($\#/\mu\text{m}^2$), IMCL area density (%), and IMCL-mitochondrial apposition both as percent IMCL touching mitochondria (%) and length of apposition (nm) in the SS, central IMF and peripheral IMF was assessed. For the length of apposition analysis only those IMCL in contact with mitochondria were included in the analysis. The SS area was defined as the space below the subsarcolemmal membrane, until the most peripheral myofibril. The peripheral IMF was defined as the area below the SS, encompassing the three most ‘superficial’ myofibrils. The central IMF region was defined as the remaining area from the fourth most ‘superficial’ myofibril inwards. Solely IMF images were taken strictly in the central IMF region. IMCL utilization was determined and expressed as an absolute change (IMCL area density pre – IMCL area density post) and percent change (absolute change/IMCL area density pre)*100%. Additionally, fibre typing was performed on each image by analysis of three non-adjacent z-discs and/or m-band width. Cutoffs for each fibre type is as detailed by Payne et al in 1975¹⁴⁶. 90nm z-discs served as a cutoff for type I muscle fibres, whereas below 80nm served as a threshold for definitively type II muscle fibres. If z-discs

measured between 80 and 90nm, the same analysis was performed on the m-bands, where >90nm served as a cutoff as type I muscle fibres. The analysis is demonstrated in Figure 3.3 and 3.4.

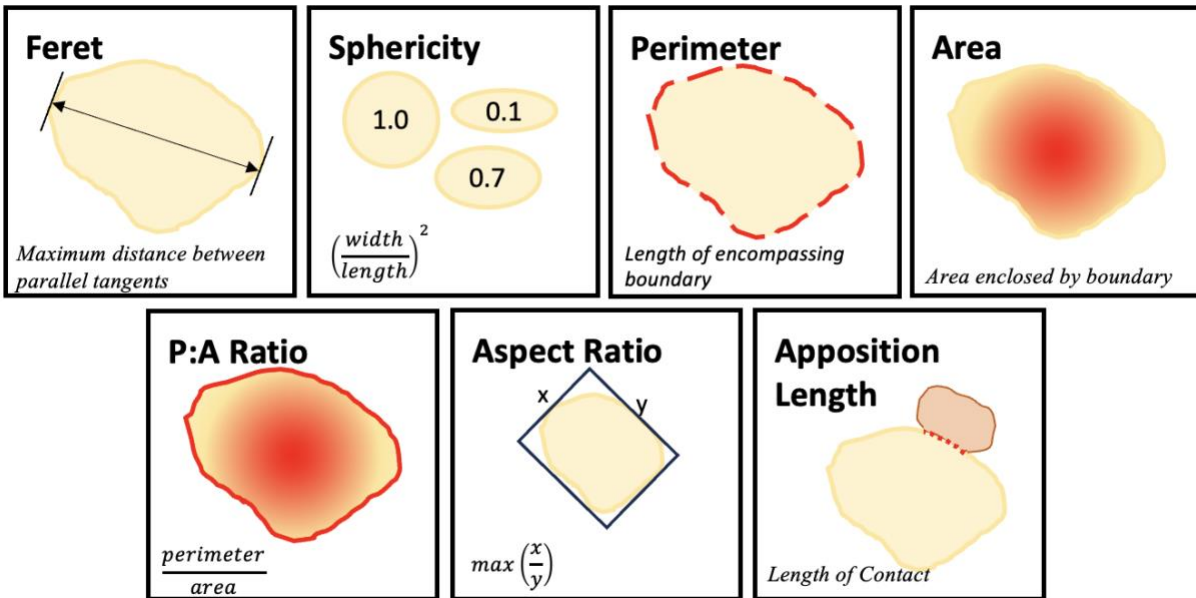


Figure 3.3. Measures of IMCL morphology assessed and respective parameters. Adapted from de Almeida (2023)¹⁴⁷.

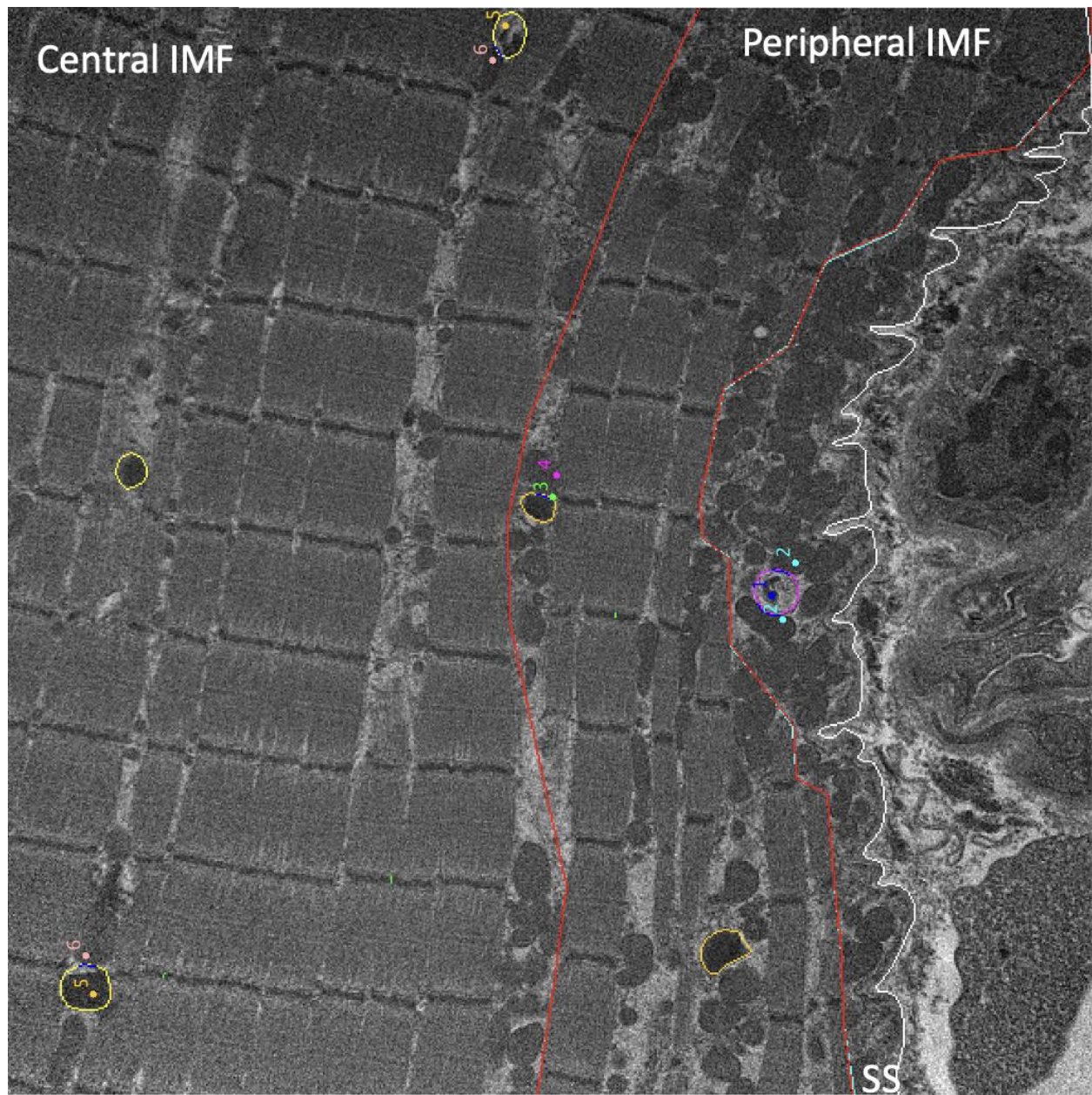


Figure 3.4. A representative image denoting the analysis performed using TEM. The vertical lines designate sectioning of the subcellular regions (L-R): central intermyofibrillar, peripheral intermyofibrillar, and subsarcolemmal. The yellow, orange, and pink circles have been placed to highlight IMCL in the central intermyofibrillar, peripheral intermyofibrillar, and subsarcolemmal areas. Counters numbered 2, 4, and 6 have been placed to highlight mitochondria in contact with IMCL, while counters 1, 3 and 5 have been placed to highlight IMCL in contact with mitochondria. Green lines crossing z-discs have been placed to highlight fibre typing via z-disc width.¹⁴²

3.3 Statistical analyses

All statistical analyses were performed on R programming software (Version 4.2.2, R Core Team, R Foundation for Statistical Computing, Vienna, Austria). Unpaired two-tailed t-tests were used to determine differences in baseline characteristics between the sexes. It was additionally used to determine differences between the sexes for average oxygen consumption, rates of fat oxidation, target HR, average RPE, HR, and lactate during both high and low intervals, the change and % change in IMCL content during exercise and differences in content of lipid and aerobic metabolism enzymes. A two-way mixed model ANOVA was used to determine the effect of sex and the acute exercise bout on all other experimental variables assessed. Both the between variable (sex) and within variable (time) had two levels: male and female, or before and after exercise, respectively. Tukey's HSD post-hoc tests were employed upon the result of a significant interaction. Significance was set to $p < 0.05$.

Chapter Four: Results

4.0 Participant Characteristics and Baseline Data

Participant characteristics and baseline data are reported in Table 4.1. There was no difference in age ($p=0.46$) or BMI ($p=0.45$) between males and females at baseline. Males were significantly taller ($p=0.0001$), had lower % body fat ($p<0.0001$) and had greater body mass ($p=0.002$) than females. Males had a greater VO_{2peak} relative to body mass ($p<0.001$) than females; however, when VO_{2peak} was calculated relative to fat-free mass and fat-free lower body mass (LB FFM), there was no significant difference between the sexes ($p=0.19$ and $p=0.27$, respectively). A comparison of habitual dietary intake data was also performed. Males consumed a greater amount of energy ($p=0.01$), as well as greater absolute amounts of protein ($p=0.02$), and fat (0.02), yet CHO intake was not significantly different ($p=0.07$). Females consumed significantly less saturated ($p=0.03$), monounsaturated ($p=0.001$), and polyunsaturated fats ($p=0.004$) compared with males. However, when expressed relative to bodyweight, daily caloric consumption did not vary between the sexes ($p=0.38$). Additionally, the percent contribution of protein ($p=0.43$), fat ($p=0.33$) CHO ($p=0.43$) and saturated ($p=0.96$), monounsaturated ($p=0.23$) and polyunsaturated ($p=0.23$) fat to energy intake did not differ between the sexes. Furthermore, relative protein intake per day was similar between the sexes ($p=0.26$), as was the contribution of saturated fats ($p=0.63$), monounsaturated fats ($p=0.16$) and polyunsaturated fats ($p=0.26$) when expressed relative to body weight.

Table 4.1: A comparison of baseline characteristics and dietary intake for participants.

	Males	Females	P-Value
Demographic Characteristics			
Age (years)	22±2	21±1	0.46
Height (cm)	177.9±2.0	162.7±2.5	0.0001
Weight (kg)	75.6±2.4	62.6±3.1	0.002
BMI (kg/m ²)	23.7±0.8	22.9±0.6	0.45
%Body Fat	20.9±1.6	32.9±1.3	<0.0001
Fitness Indices			
VO2peak (ml O ₂ /min/kg)	43.1±1.6	34.4±1.4	<0.001
VO2peak (ml O ₂ /min/kg FFM)	59.5 ±1.5	56.5±1.7	0.20
VO2peak (ml O ₂ /min/kg LB FFM)	180.1±11.4	166.7±4.4	0.28
Daily Activity (steps/day)	8307±466	9679±856	0.22
Muscle Fibre Type			
Type I	50.0±5.6	55.6±5.4	0.45
Type II	50.0±5.6	44.4±5.4	0.45
Dietary Intake			
Daily energy intake (kcal)	2008±171	1477±78	0.01
Relative energy intake (kcal/kgBW)	26.6±1.66	24.7±1.24	0.38
Protein intake			
Absolute intake (g)	96±11	65±4	0.02
Relative intake (g/kgBW/d)	1.27±0.15	1.07±0.09	0.26
% of daily energy intake (%)	19±2	17±1	0.43
Fat intake			
Absolute intake (g)	79±7	56±5	0.02
Relative intake (g/kgBW)	1.04±0.07	0.93±0.07	0.26
% of daily energy intake (%)	36±2	33±2	0.33
Saturated fat intake			
Absolute intake (g)	25.0±2.3	18.5±1.5	0.031
Relative intake (g/kgBW)	0.33±0.03	0.31±0.03	0.63
% of daily energy intake (%)	11.2±0.8	11.3±0.8	0.96
Monounsaturated fat intake			
Absolute intake (g)	30.9±2.5	20.9±1.6	0.001
Relative intake (g/kgBW)	0.40±0.03	0.34±0.03	0.16
% of daily energy intake (%)	14.2±1.0	12.6±0.9	0.23
Polyunsaturated fat intake			
Absolute intake (g)	16.1±1.4	11.3±0.9	0.004
Relative intake (g/kgBW)	0.21±0.02	0.18±0.01	0.26
% of daily energy intake (%)	7.6±0.7	6.7±0.5	0.28
CHO intake			
Absolute intake (g)	234±23	186±11	0.07
Relative intake (g/kgBW)	3.11±0.23	3.05±0.17	0.85
% of daily energy intake (%)	47±2	50±2	0.43

Data are expressed as means±SEM. Comparisons made by unpaired t-tests. BW: body weight, d: day, g: gram, kg: kilogram

4.1 Indirect Calorimetry during HIIE

4.1.1 Wattage during the acute exercise bout

Data regarding the wattage cycled at during the acute exercise bout can be found in Figure 4.1. During the acute exercise bout, the wattage corresponding to the high intensity intervals was higher in males than females ($p<0.0001$). This relationship was maintained even when the wattage was expressed relative to LB FFM ($p=0.02$). The wattage corresponding to the low intensity intervals relative to LB FFM was higher in the females compared to males ($p=0.0002$).

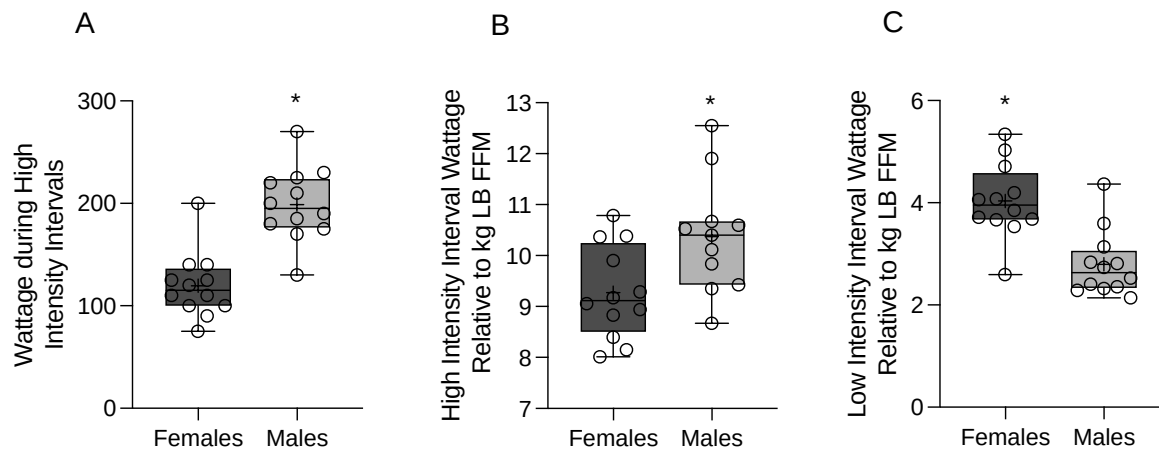


Figure 4.1: Box and whisker plots showing A) Wattage during high intensity intervals, * greater than females, $p<0.0001$; B) Wattage during high intensity intervals expressed relative to kg LB FFM, *greater than females, $p=0.02$; and C) Wattage during the low intensity intervals expressed relative to kg LB FFM, *greater than males, $p=0.0002$. The cross and line within the boxplot represent the mean and median, respectively, while the border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using unpaired T-tests. LB FFM: Lower body fat-free mass. Adapted from Woodgate (2020).

4.1.2 Rating of Perceived Exertion during the acute exercise bout

Rating of perceived exertion (RPE) data can be found in Figure 4.2. There was no difference in RPE between the sexes ($p=0.66$). There was a main effect of time by which RPE

increased during exercise ($p<0.0001$). There was no sex by time interaction for RPE ($p=0.15$).

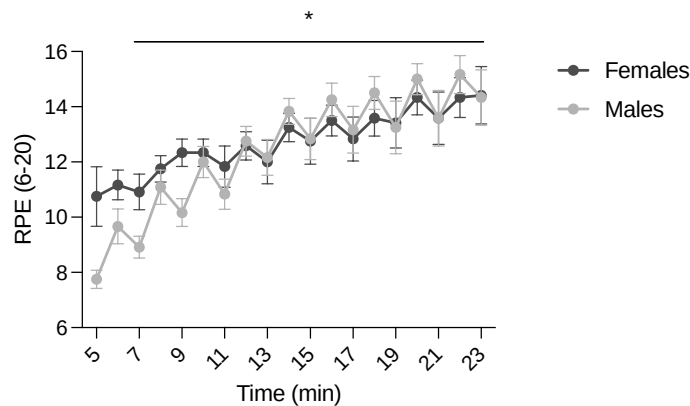


Figure 4.2: Line graph of RPE taken every minute during the exercise bout, *greater than minutes 5-7, $p<0.0001$. Data presented as mean $\pm$ SEM. Comparisons made two-way mixed model ANOVAs. Adapted from Woodgate (2020).

4.1.3 Heart rate and VO₂ during the acute exercise bout

All heart rate data collected during the acute exercise bout is shown in Figure 4.3. There was no difference in heart rate between males and females ($p=0.17$). While there was a main effect of time by which HR increased during exercise in both males and females ($p<0.0001$), there was no interaction between sex and time ($p=0.06$). Females achieved a greater % target heart rate during the high intensity intervals compared with males (females: $101\pm1\%$, males: $93\pm2\%$, $p=0.002$). Additionally, females exercised at a higher % VO₂peak during the HIIE bout as compared with males (females: $69\pm3\%$, males: $55\pm3\%$, $p=0.01$).

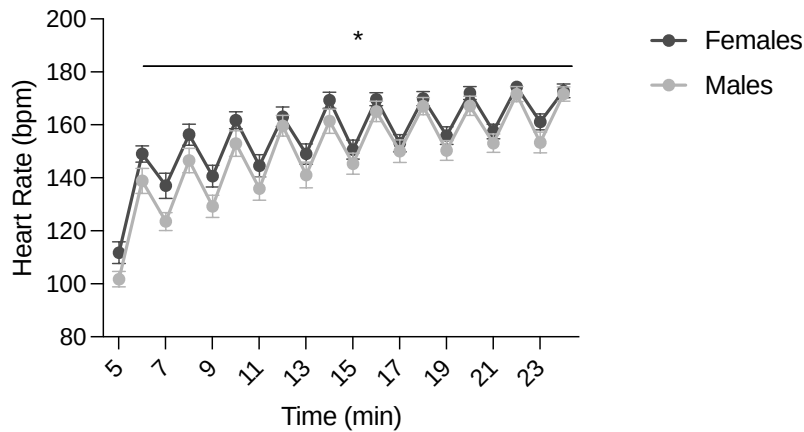


Figure 4.3: Line graph showing HR (bpm) taken each minute for males and females during the exercise bout, *greater than minute 5, $p < 0.0001$. Comparisons made from two-way mixed model ANOVAs. bpm: beats per minute. Adapted from Woodgate (2020).

4.1.4 Total Energy Expenditure during the acute exercise bout

Data regarding total energy expenditure during the acute exercise bout in males and females are reported in Figure 4.5. Overall, males had a greater total energy expenditure during the exercise bout compared to females ($p = 0.001$). However, when expressed relative to kg FFM this relationship was no longer significant ($p = 0.13$).

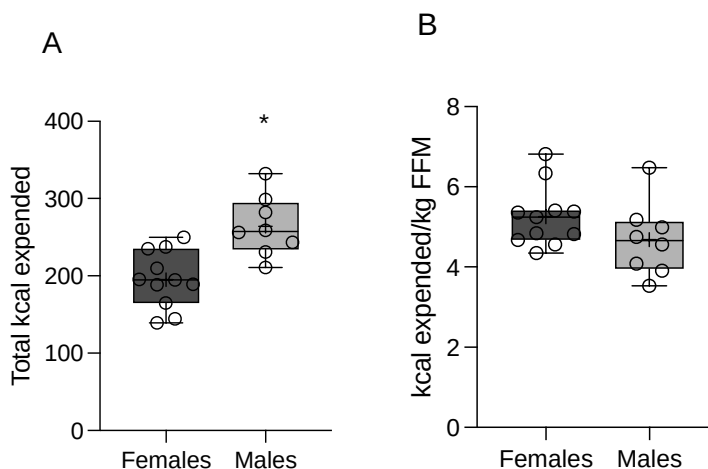


Figure 4.5: Box and whisker plots showing A) the total energy expenditure (kcal) during the high intensity interval exercise bout, *higher than females, $p = 0.001$; and B) the total energy expenditure (kcal) during the high intensity interval exercise bout expressed relative to kg FFM.

The cross within the boxplot represents the mean, and circles represent individual participant values. The line within the boxplot represents the median, while the border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using unpaired T-tests. FFM: Fat free mass, kcal: kilocalorie. Adapted from Woodgate (2020).

4.1.5 Whole Body Substrate Utilization during the acute exercise bout

4.1.5.1 RER, fat and CHO oxidation during the acute exercise bout

The data for RER during the acute exercise bout is reported in Table 4.4. There was no difference in the RER between males and females ($p = 0.29$). The RER was greater during the high intensity and low intensity intervals than the warmup and cool down ($p < 0.0001$) and greater during the cool down than during the warm-up ($p < 0.0001$). There was no difference in the RER during any of the exercise stages between males and females ($p = 0.55$).

Data for the % contribution of fat and CHO oxidation to energy expenditure is reported in Table 4.3 and Figure 4.6. There was no effect of sex on %fat or %CHO contribution to TEE ($p = 0.56$ for both). There was a significant effect of time, by which the % contribution of fat was greater during the warmup than at any other point during the exercise bout ($p < 0.0001$). Conversely, the significant main effect of time for %CHO contribution to TEE indicated that it was higher at all time points during exercise than during warmup ($p < 0.0001$). There was no significant sex x time interaction for %fat or %CHO contribution to TEE ($p = 0.35$ for both).

The results for the rate of fat and CHO oxidation can also be found in Table 4.3. There was no main effect of sex on the rate of fat oxidation ($p = 0.98$). There was a significant main effect of time for the rate of fat oxidation ($p < 0.0001$), by which it was higher during the warm-up than any other timepoint during exercise ($p < 0.0001$). Furthermore, the rate of fat oxidation was higher during the high intensity intervals ($p = 0.02$) and during the cool-down ($p = 0.006$) as compared with during the low intensity intervals. There was a main effect of sex on the rate of CHO oxidation by which males had a greater rate of CHO oxidation than females ($p < 0.0001$). There

was also a significant main effect of time by which the rate of CHO oxidation was greater during both the high and low intensity intervals than during the warmup and cool down ($p < 0.0001$). Similar to fat oxidation rates, CHO oxidation rates were also lower in the cool down than during the warm-up ($p < 0.0001$). Opposite to fat oxidation, the rate of CHO oxidation was lower in the high intervals compared with the low intervals ($p = 0.002$). A significant sex x time interaction occurred in that males had a higher rate of CHO oxidation during all stages of exercise except for the warm-up ($p = 0.001$ high interval, $p < 0.0001$ low interval, $p = 0.001$ cool down, $p = 0.65$ warm-up) compared with females.

Table 4.3: RER, % CHO and % Fat Contribution to total energy expenditure and rate of CHO and fat oxidation during the HIIE bout in males and females.

	Males	Females	P-Value		
			Sex	Time	Sex x Time
RER			0.55	<0.0001	0.29
Warmup	0.84±0.02	0.86±0.02			
High Intervals	0.99±<0.01	0.97±0.01			
Low Intervals	0.99±<0.01	0.98±0.01			
Cool Down	0.96±0.01	0.93±0.01			
%Fat Contribution to TEE			0.56	<0.0001	0.31
Warmup	50.8±7.8	45.3±7.6			
High Intervals	4.6±1.5	8.2±1.9			
Low Intervals	3.2±1.1	6.5±2.1			
Cool Down	14.1±3.2	23.0±3.9			
%CHO Contribution to TEE			0.56	<0.0001	0.31
Warmup	49.2±7.8	54.7±7.6			
High Intervals	95.4±1.5	91.8±1.9			
Low Intervals	96.8±1.1	93.5±2.1			
Cool Down	85.9±3.2	77.0±3.9			
Rate of Fat Oxidation (g/min)			0.98	<0.0001	0.17
Warm-Up	0.27±0.03	0.22±0.04			
High Intervals	0.05±0.02	0.07±0.02			
Low Intervals	0.04±0.01	0.03±0.01			
Cool Down	0.10±0.02	0.15±0.03			
Rate of CHO Oxidation (g/min)			<0.0001	<0.0001	<0.0001
Warm-Up	0.61±0.10	0.56±0.07			
High Intervals	2.35±0.14	1.69±0.10			
Low Intervals	2.63±0.13	1.69±0.10			
Cool Down	1.39±0.09	1.02±0.04			

Data are expressed as mean±SEM. P values are expressed as main effect of sex, main effect of time across the exercise bout and the interaction between sex and time, respectively.

Comparisons made two-way mixed model ANOVAs. RER: respiratory exchange ratio, TEE: total energy expenditure, CHO: CHO. Adapted from Woodgate (2020).

Data for the absolute and relative amounts of fat and CHO oxidized during the exercise bout are reported in Table 4.4. There was no effect of sex on total grams of fat oxidized across the acute exercise bout as a whole ($p=0.94$), relative to body weight ($p=0.49$) or relative to fat-free mass ($p=0.19$). There was a main effect of time on the amount of fat oxidized ($p<0.0001$) indicating that it was lower at all timepoints during exercise compared with during the warm-up.

Additionally, the amount of fat oxidized was lower during the low intensity intervals than during the high intensity intervals ($p=0.016$) and the cool down ($p=0.006$). There were no interactions between sex and time for the absolute or relative amount of fat oxidized ($p=0.10$, $p=0.13$, $p=0.12$, respectively).

There was a significant main effect of sex by which males oxidized a greater total amount of CHO ($p<0.001$) across the entire exercise bout, which persisted when expressed relative to bodyweight ($p<0.05$), but not when expressed relative to fat-free mass ($p=0.40$). There was a main effect of time by which total amount of CHO oxidized was lower in the high intensity intervals than during the low intervals ($p=0.002$). There was a significant sex by time interaction by which males oxidized more CHO in the high intensity, low intensity, and cool down intervals ($p=0.001$, $p<0.0001$, $p=0.001$, respectively) but this was not the case during the warm-up ($p=0.65$). Additionally, the interaction by which males oxidized more CHO during the low intensity intervals persisted when expressed relative to body weight ($p=0.001$), but not when expressed relative to FFM ($p=0.26$).

Table 4.4: Absolute and Relative amount of Fat and CHO Oxidized during the Warm-Up, High Intervals, Low Intervals, and Cool down.

	Males	Females	P Value		
			Sex	Time	Sex x Time
Fat (g)			0.94	<0.0001	0.1
Warm-Up	1.3±0.2	1.1±0.2			
High Intervals	0.5±0.2	0.7±0.2			
Low intervals	0.4±0.1	0.3±0.1			
Cool Down	0.5±0.1	0.7±0.1			
g Fat/kgBW			0.49	<0.0001	0.13
Warm-Up	0.02±0.01	0.02±<0.01			
High Intervals	<0.01±<0.01	0.01±<0.01			
Low intervals	0.01±<0.01	<0.01±<0.01			
Cool Down	0.01±<0.01	0.01±<0.01			
g Fat/kgFFM			0.19	<0.0001	0.12
Warm Up	0.02±<0.01	0.03±<0.01			
High Intervals	0.01±<0.01	0.02±<0.01			
Low intervals	0.01±<0.01	0.01±0.01			
Cool Down	0.01±0.01	0.02±0.01			
CHO (g)			<0.0001	<0.0001	<0.0001
Warm-Up	3.1±1.6	2.8±0.3			
High Intervals	23.5±1.4	16.9±1.0			
Low intervals	26.3±1.3	16.9±0.9			
Cool Down	7.0±0.4	5.1±0.2			
g CHO/kgBW			0.046	<0.0001	0.02
Warm-Up	0.04±0.01	0.05±0.01			
High Intervals	0.31±0.02	0.27±0.01			
Low intervals	0.34±0.02	0.27±0.01			
Cool Down	0.09±<0.01	0.8±0.01			
g CHO/kgFFM			0.4	<0.0001	0.26
Warm-Up	0.05±0.01	0.08±0.01			
High Intervals	0.42±0.03	0.46±0.02			
Low intervals	0.47±0.03	0.45±0.02			
Cool Down	0.12±0.01	0.14±0.01			

Data are expressed as mean±SEM. P values are expressed as main effect of sex, main effect of time across the exercise bout and the interaction between sex and time, respectively.

Comparisons made using two-way mixed model ANOVAs. CHO: carbohydrates, g: grams, kgBW: kilograms body weight, kgFFM: kilograms fat-free mass

Data for the total amount of fat and CHO oxidized during the acute HIIE bout are reported in Table 4.5. There was no difference in the total amount of fat oxidized between males and females when expressed as total grams oxidized, grams oxidized relative to body weight or grams oxidized relative to FFM ($p=0.94$, $p=0.49$, $p=0.19$, respectively). Males oxidized more CHO during the acute exercise bout than females when expressed as grams oxidized ($p<0.0001$) and grams oxidized relative to body weight ($p=0.03$), but not when expressed as grams oxidized relative to FFM ($p=0.89$).

Table 4.5 Absolute and Relative amount of Fat and CHO oxidized during the Total Exercise Bout

	Males	Females	P-Value
Fat			
Fat (g)	2.8±0.5	2.8±0.5	0.94
g Fat/kgBW	0.04±0.01	0.04±0.01	0.49
g Fat/kgFFM	0.05±0.01	0.07±0.01	0.19
CHO			
CHO (g)	63.6±4.1	41.7±1.8	<0.0001
g CHO/kgBW	0.84±0.07	0.68±0.03	0.03
g CHO/kgFFM	1.14±0.12	1.27±0.05	0.89

Data are expressed as mean±SEM. Comparisons made using unpaired T-tests. g: grams, kgBW: kilograms body weight, kgFFM: kilograms fat-free mass.

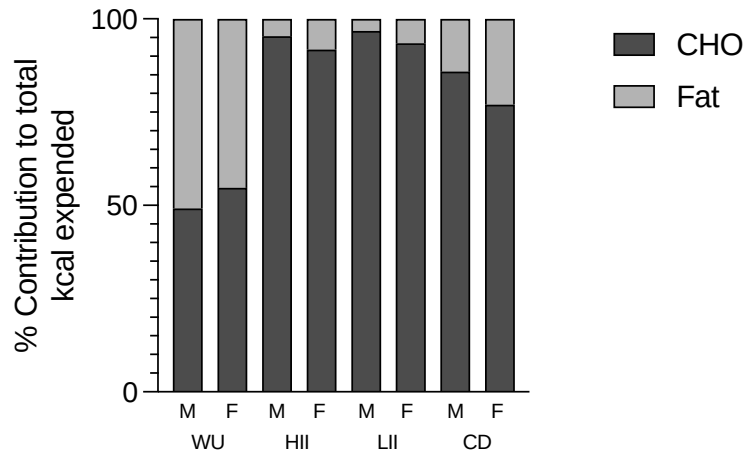


Figure 4.6: %Fat and %CHO contribution to total energy expenditure during each segment of the HIIE bout. CHO: Carbohydrate, WU: Warm-up, HII: High intensity interval, LII: Low intensity interval, CD: Cool down. Adapted from Woodgate (2020).

4.2 Blood Lactate Concentration during the acute exercise bout

Blood lactate data during the acute exercise bout in males and females are reported in Figure 4.7. There was no effect of sex on blood lactate concentration ($p=0.35$). There was a main effect of time indicating that blood lactate increased across the exercise bout ($p<0.0001$). Additionally, there was a significant interaction indicating that while blood lactate concentrations were higher in females after the warm-up ($p=0.007$). However, the increase in blood lactate during the exercise bout was greater in males than females ($p=0.02$).

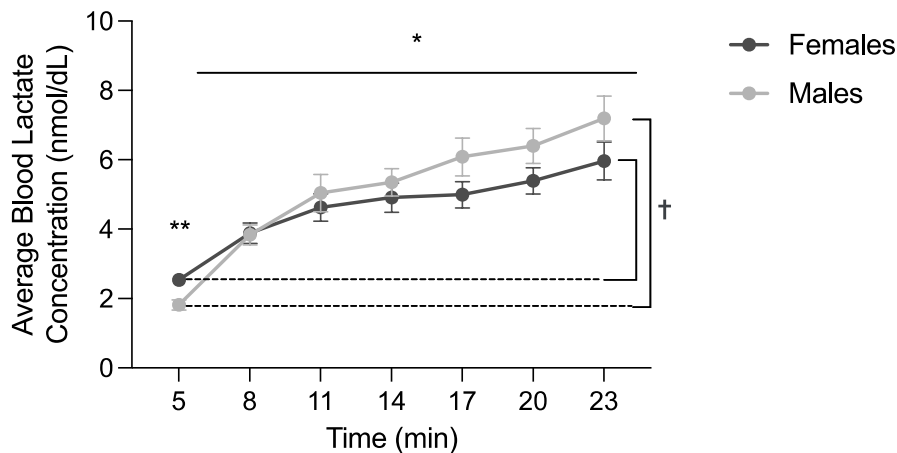


Figure 4.7: A line graph showing blood lactate concentrations during the exercise bout *greater than the end of warm up, $p < 0.0001$, **greater in females, $p = 0.0007$, †greater in males, $p = 0.02$; Comparisons were made using two-way mixed model ANOVAs. Data are reported as mean $\pm$ SEM.

4.3 Western Blot Results

Western blot analysis was conducted on the biopsies collected prior to exercise.

Representative images of the standard curves and stain-free blots involved in the normalization process can be found in the Appendix (Supplementary Images 1-4)

4.3.1 Content of IMCL Turnover Proteins

Content of proteins involved in IMCL turnover are shown in Figure 4.8. There was no significant difference in the content of PLIN2 ($p = 0.45$), PLIN 3 ($p = 0.36$), or PLIN 5 ($p = 0.08$) between males and females.

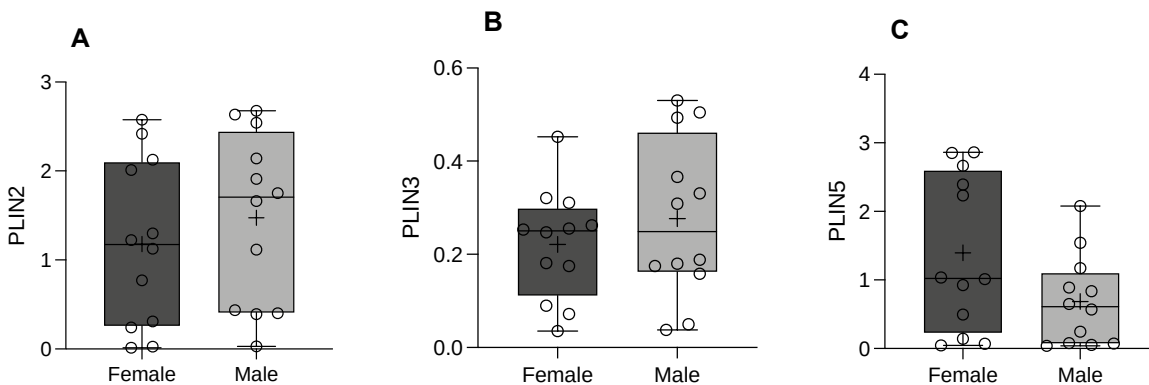


Figure 4.8: Boxplots of A) PLIN2, B) PLIN3, and C) PLIN5 in *vastus lateralis* samples from males ($n = 12$) and females ($n = 12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using unpaired T-tests.

4.3.2 Content of Lipid Metabolism Proteins

Content of proteins involved in lipid metabolism are shown in Figure 4.9. There was no significant difference in the content of ATGL ($p=0.10$), CPT1 ($p=0.29$), or SCHAD ($p=0.79$) between males and females.

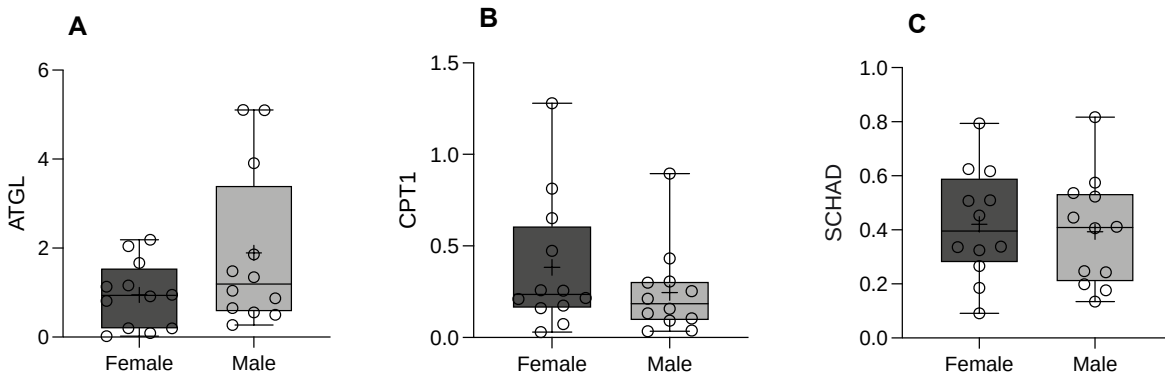


Figure 4.9: Boxplots of A) ATGL, B) CPT1, and C) SCHAD in *vastus lateralis* samples from males ($n=12$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using unpaired T-tests.

4.3.3 Content of Aerobic Metabolism Proteins

Content of proteins involved in aerobic metabolism are shown in Figure 4.10. There was no significant difference in content of Complex I ($p=0.24$), Complex II ($p=0.41$), Complex III ($p=0.08$), Complex IV ($p=0.15$) or Complex V ($p=0.42$) or citrate synthase ($p=0.46$) between males and females.

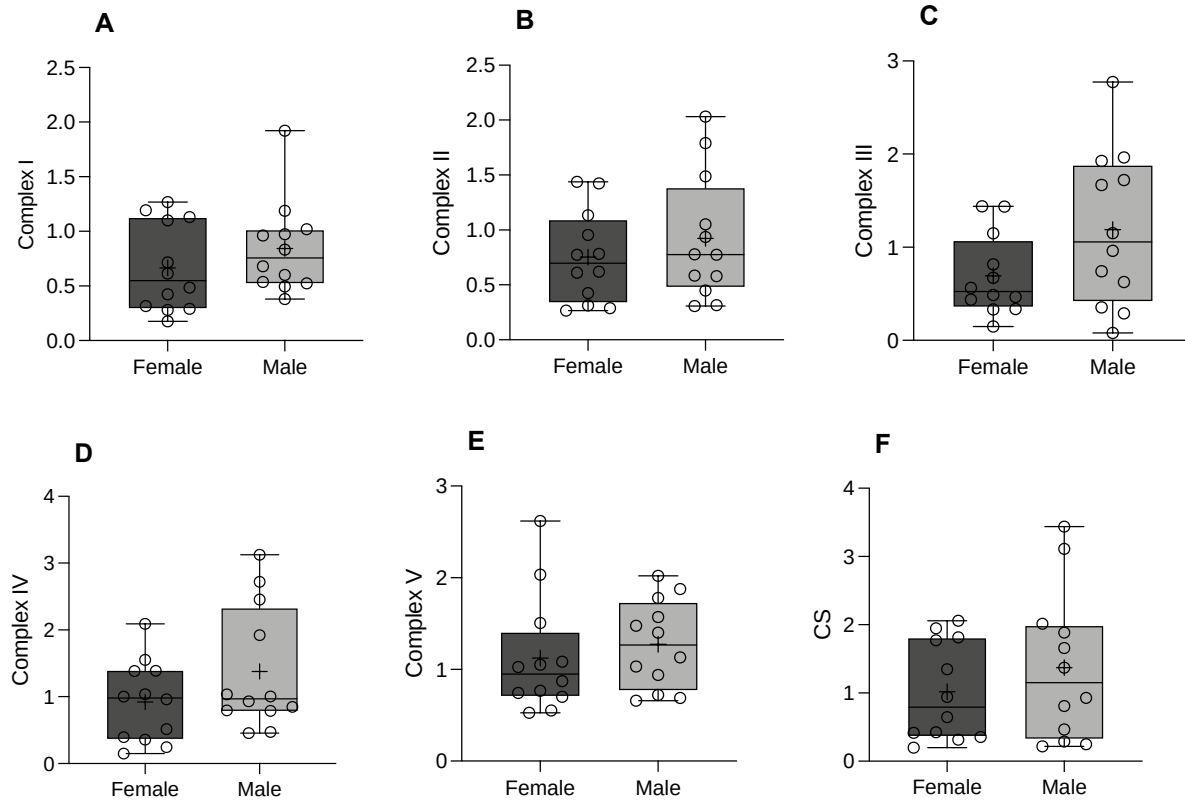


Figure 4.10: Boxplots of A) Complex I, B) Complex II, C) Complex III, D) Complex IV, E) Complex V, F) CS in *vastus lateralis* samples from males (n=12) and females (n=12). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using unpaired T-tests.

4.4 Transmission Electron Microscopy

Representative electron micrographs of males and females prior to and following exercise can be found in Figure 4.11. Muscle samples from twenty-three subjects consisting of eleven males and twelve females were used for transmission electron microscopy analysis. A pre and post exercise biopsy from one male, and pre biopsy for one female are missing because insufficient muscle was obtained during the biopsy.

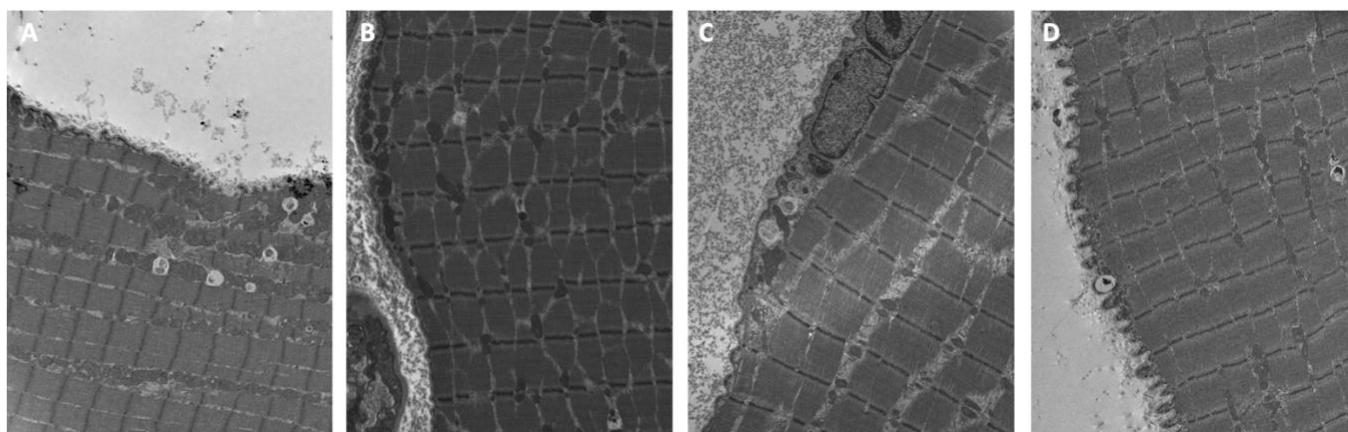


Figure 4.11. Representative electron micrographs from one female prior to (A) and following exercise (B) and one male prior to (C) and following exercise (D).

Data regarding IMCL size is noted in Figure 4.12 and Table 4.6. There was a significant main effect of sex for IMCL size for all muscle areas such that IMCL size was larger in males in the SS ($p=0.003$), peripheral IMF ($p=0.0004$), and central IMF ($p=0.04$), as well as averaged across all areas ($p=0.0006$). There was no significant effect of exercise on IMCL size in the SS ($p=0.61$), peripheral IMF ($p=0.18$) or central IMF ($p=0.51$) areas, nor across the total area ($p=0.47$). There was no sex x time interaction for IMCL size in the SS ($p=0.81$), peripheral IMF ($p=0.98$) or central IMF ($p=0.09$) areas, nor across the total areas ($p=0.77$). There was a significant difference in absolute change in IMCL size between the sexes in the central IMF ($p=0.03$) where it decreased in females but increased in males, but not in any other area of the myocyte [SS ($p=0.81$), peripheral IMF ($p=0.98$), or total area ($p=0.97$)]. There was no significant differences in % change in IMCL in any area of the myocyte [SS ($p=0.65$), peripheral IMF ($p=0.38$), central ($p=0.07$), or total area ($p=0.72$)].

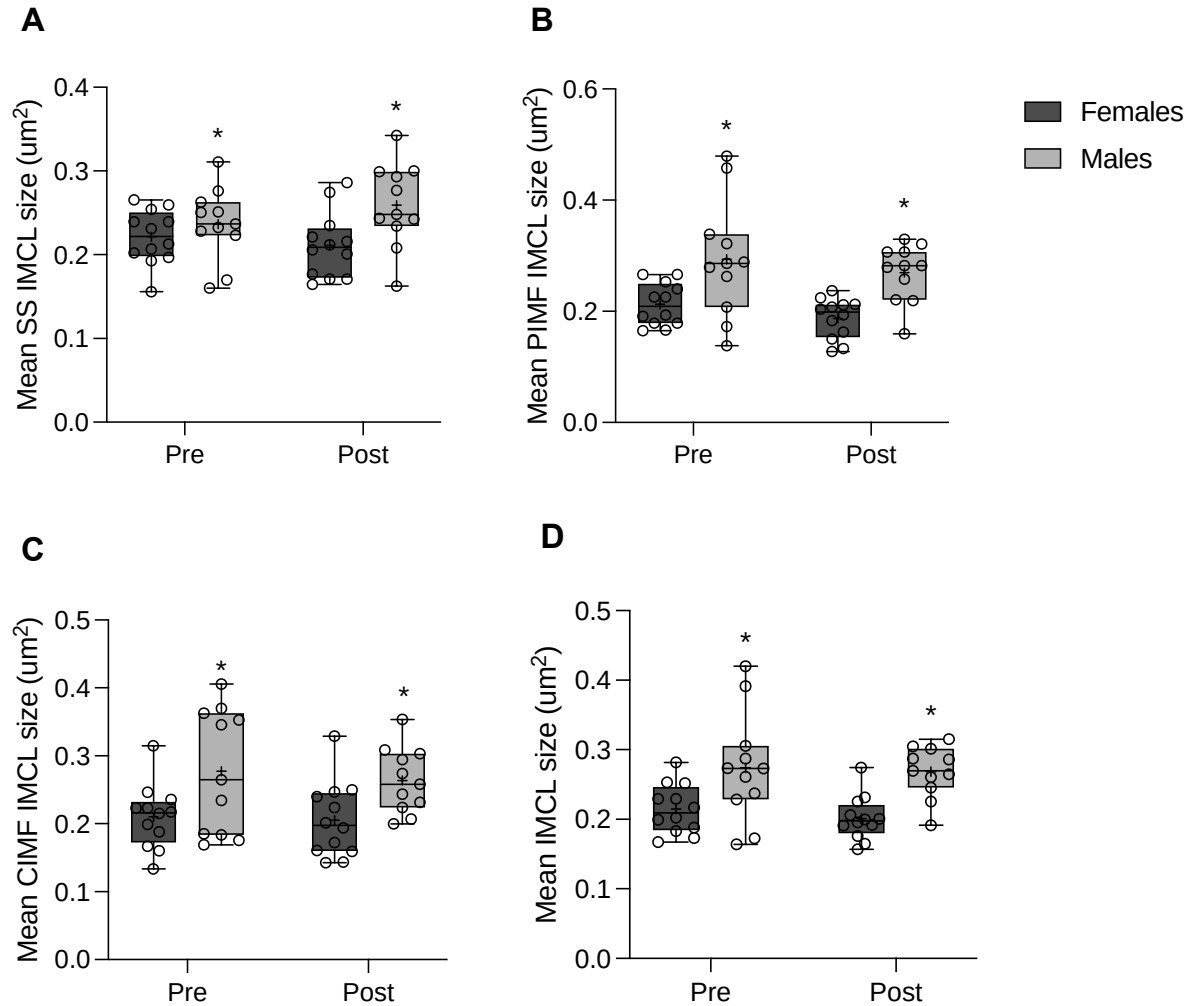


Figure 4.12: Boxplots representing average IMCL size (um²) in the A) SS region, *larger than females, $p=0.003$, B) peripheral IMF, *larger than females, $p=0.0004$ C) central IMF, *larger than females, $p=0.04$ and D) averaged across all regions, *larger than females, $p=0.0006$ prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using unpaired two-way, mixed model ANOVAs.

Table 4.6. Change and %Change of IMCL size across the HIIE Bout

	Males	Females	P-Value
SS			
Change	-0.014±0.033	-0.005±0.017	0.81
%Change	6.7±13.5	-0.2±7.4	0.65
Peripheral IMF			
Change	-0.024±0.034	-0.025±0.014	0.98
% Change	-7.7±9.0	-9.7±6.7	0.86
Central IMF			
Change	0.023±0.016	-0.018±0.008	0.03
% Change	11.4±6.6	-3.8±5.0	0.07
Total area			
Change	-0.006±0.025	-0.006±0.008	0.97
% Change	-3.6±9.8	-2.5±3.9	0.91

Data are expressed as mean±SEM. Comparisons made using unpaired T-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding the number of IMCL in a given area ($\#/um^2$) is noted in Figure 4.13 and Table 4.7. There was a significant main effect of sex on the number of IMCL in the SS ($p=0.03$), peripheral IMF ($p<0.0001$) and total muscle ($p=0.002$) areas, but not in the central IMF area ($p=0.86$), indicating females had a greater number of IMCL in these areas. There was no effect of exercise on number of IMCL in any region [SS ($p=0.43$), peripheral IMF ($p=0.33$), central IMF ($p=0.25$), total ($p=0.99$) area]. There was also no interaction between sex and exercise on IMCL number [SS ($p=0.82$), peripheral IMF ($p=0.66$), central IMF ($p=0.70$), total ($p=0.95$) areas]. There were no significant differences in the absolute change in IMCL number between the sexes in any region of the myocyte [SS ($p=0.75$), peripheral IMF ($p=0.15$), central IMF ($p=0.43$) or total area ($p=0.75$)]. There were no significant differences in the %change in IMCL

number between the sexes in any area of the myocyte [SS ($p=0.87$), peripheral IMF ($p=0.98$), central IMF ($p=0.17$) or total area ($p=0.31$)].

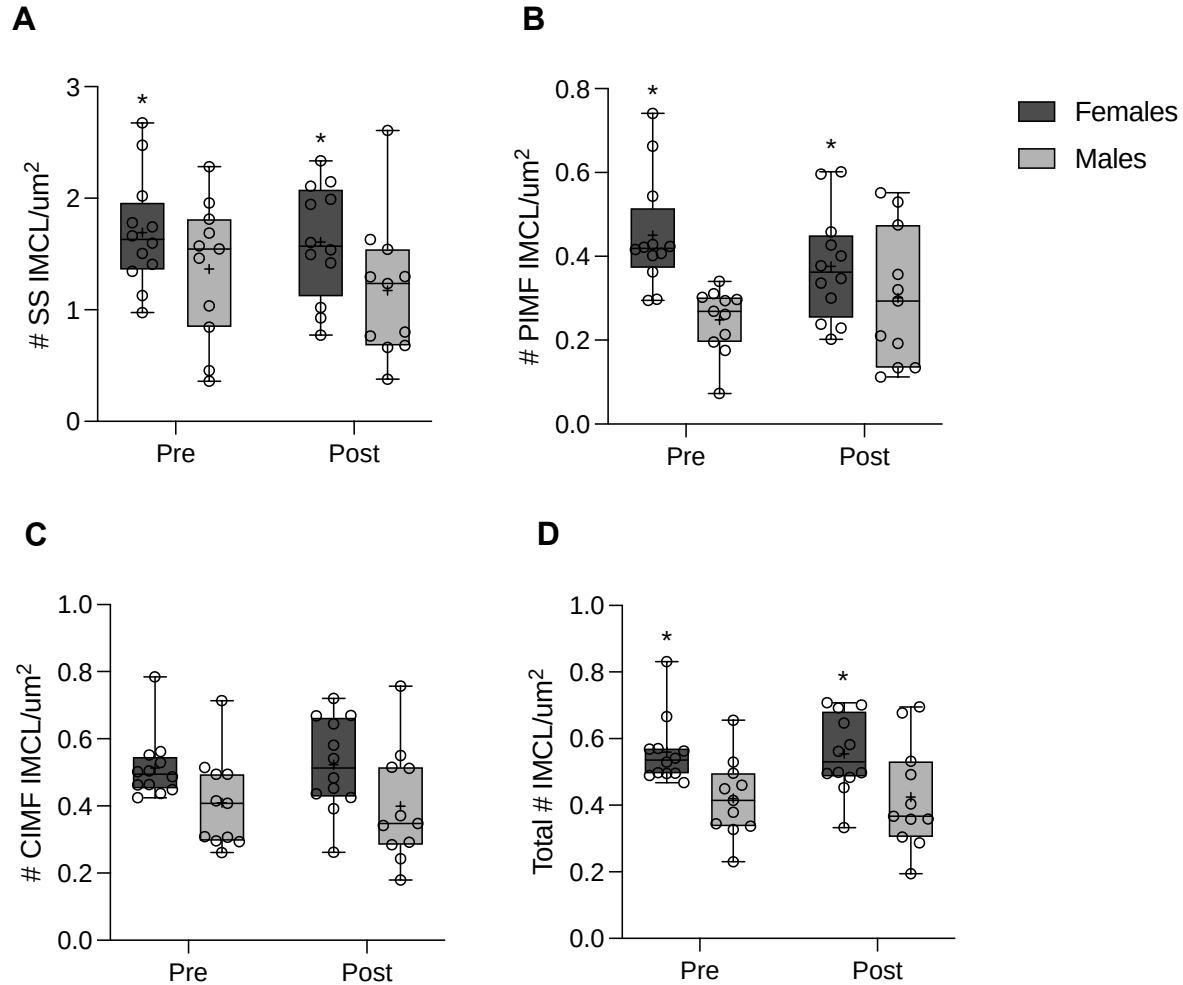


Figure 4.13: Boxplots representing the average number of IMCL in the A) SS region, *greater than males, $p=0.03$ B) peripheral IMF, *greater than males, $p<0.0001$ C) central IMF and D) total area, *greater than males, $p=0.002$ prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way, mixed model ANOVAs.

Table 4.7. Change and %Change in #IMCL across the HIIE Bout

	Males	Females	P-Value
SS			
Change	-0.193±0.277	-0.084±0.208	0.75
%Change	-2.2±24.9	2.0±11.8	0.87
Peripheral IMF			
Change	0.053±0.046	-0.034±0.036	0.15
% Change	-5.1±20.9	-5.6±8.9	0.98
Central IMF			
Change	-0.010±0.050	0.041±0.040	0.43
% Change	-13.2±12.7	7.7±8.1	0.17
Total area			
Change	0.004±0.053	0.024±0.034	0.75
% Change	-9.5±12.4	4.3±5.9	0.31

Data are expressed as mean±SEM. Comparisons were made using unpaired T-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding the average IMCL % area density is found in Figure 4.14 and Table 4.8 Sex did not effect IMCL %area density in any region of the myocyte [SS (p=0.58), peripheral IMF (p=0.31), central IMF (p=0.46) or total (p=0.34) area]. Exercise also did not effect the IMCL %area density in any region of the myocyte [SS (p=0.61), peripheral IMF (p=0.37), central IMF (p=0.97) or total (p=0.88) area]. There was no interaction of sex and exercise on IMCL %area density in any region of the myocyte [SS (p=0.94), peripheral IMF (p=0.47), central IMF (p=0.67) or total (p=0.67) area]. There were no significant differences in the absolute change in IMCL area density between the sexes in any region of the myocyte [SS (p=0.51), peripheral IMF (p=0.47), central IMF (p=0.65) or total area (p=0.67)]. There were no significant differences in

the %change between the sexes in any area of the myocyte [SS ($p=0.49$), peripheral IMF ($p=0.09$), central IMF ($p=0.64$) or total area ($p=0.67$)].

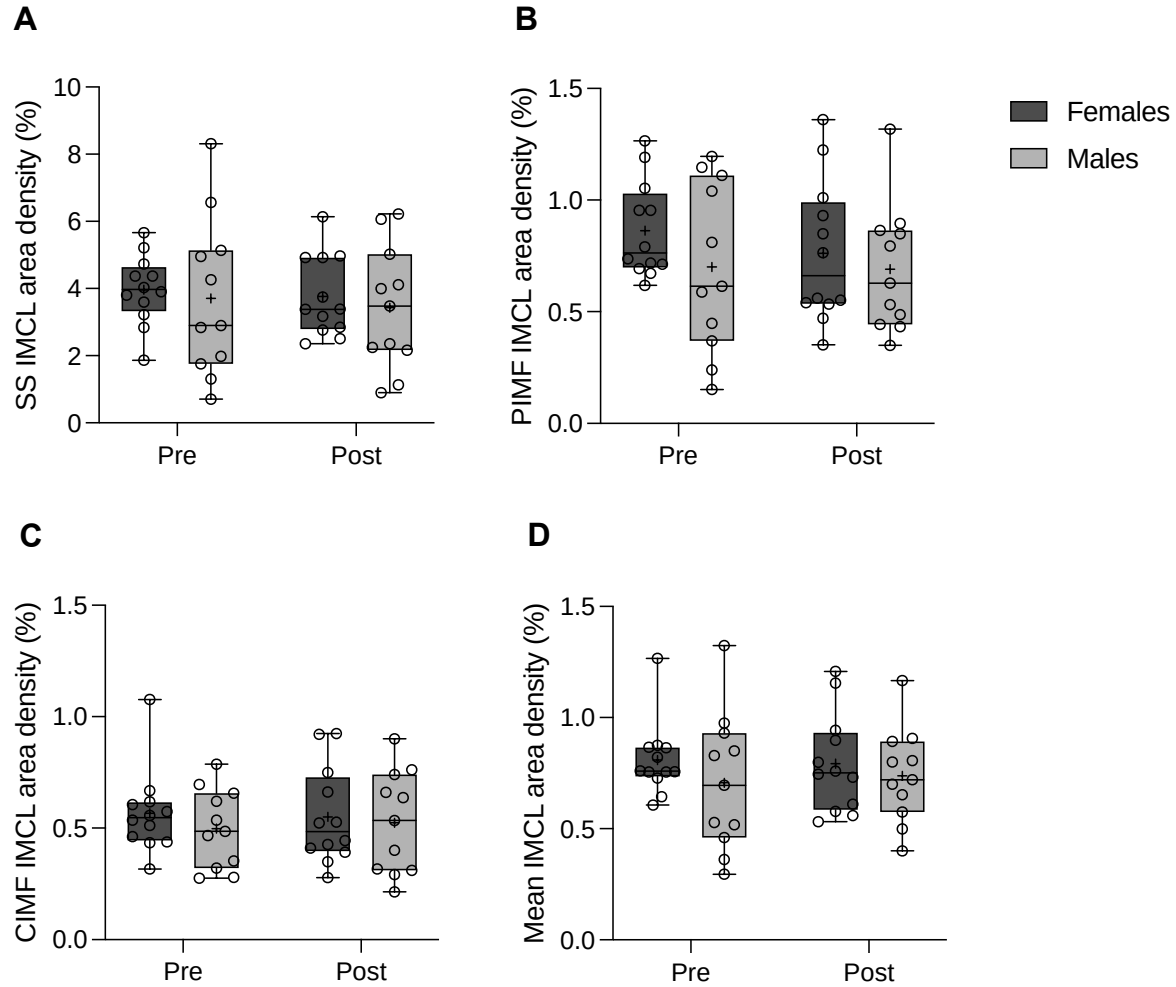


Figure 4.14: Boxplots representing IMCL area density (%) in the A) SS region, B) peripheral IMF, C) central IMF, and D) total area prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way mixed model ANOVAs.

Table 4.8. Change and %Change in IMCL Area Density (%) across the HIIIE Bout

	Males	Females	P-Value
SS			
Change	-0.31±0.64	-0.21±0.46	0.51
%Change	16.5±21.3	1.1±10.8	0.49
Peripheral IMF			
Change	-0.01±0.08	-0.10±0.09	0.47
% Change	24.5±22.9	-18.5±7.5	0.09
Central IMF			
Change	0.02±0.07	-0.02±0.07	0.65
% Change	11.6±16.3	2.0±12.2	0.64
Total area			
Change	0.03±0.09	-0.02±0.07	0.67
% Change	4.8±9.6	-0.4±7.8	0.67

Data are expressed as mean±SEM. Comparisons were made using unpaired t-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding the length of IMCL feret is noted in Figure 4.15 and Table 4.9. The IMCL feret was wider in males than females in the peripheral IMF ($p<0.001$) and total ($p=0.02$) area, and tended to be wider in males in the SS region ($p=0.07$), but did not differ between the sexes in the CIMF area ($p=0.50$). There was no significant effect of exercise on IMCL feret in any region [SS ($p=0.39$), peripheral IMF ($p=0.15$), central IMF ($p=0.44$), total ($p=0.15$) area]. Interestingly, while there was no significant interaction in the SS ($p=0.29$), peripheral IMF ($p=0.15$) or total ($p=0.19$) area, there was a significant interaction in the central IMF ($p=0.02$). Decomposition revealed that while there was no difference between the sexes prior to exercise ($p=0.41$), feret was significantly wider in males than females post-exercise ($p=0.03$). This may be in part due another non-significant interaction whereby feret tended to decrease in females post-exercise

($p=0.07$), but stayed the same in males ($p=0.28$). There was a significant difference in absolute change of IMCL feret in the CIMF ($p=0.03$) whereby the value increased in males but decreased in females. There were no significant differences in absolute change in IMCL feret between the sexes in any other region of the myocyte [SS ($p=0.78$), peripheral IMF ($p=0.15$), or total area ($p=0.19$)]. There was a significant difference in %change of IMCL feret in the CIMF ($p=0.03$), whereby it increased also in males but decreased in females. There were no significant differences in %change in IMCL feret between the sexes in any other area of the myocyte [SS ($p=0.61$), peripheral IMF ($p=0.19$), or total area ($p=0.30$)].

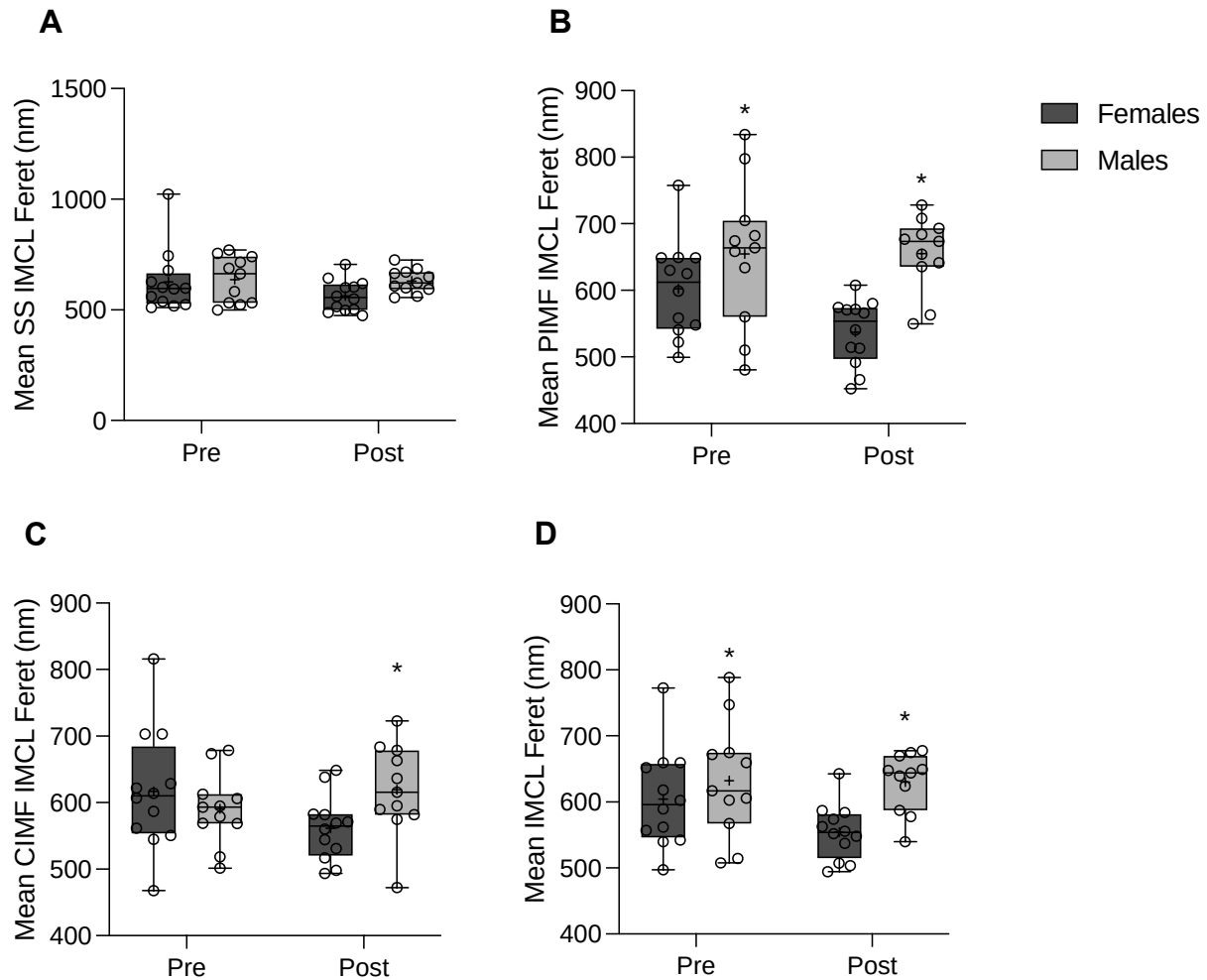


Figure 4.15: Boxplots representing average IMCL feret in the A) SS region, B) peripheral IMF, * greater than females, $p<0.001$ C) central IMF, *greater than females post-exercise, $p=0.03$ D) and total area, *greater than females, $p=0.02$ prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way, mixed model ANOVAs.

Table 4.9. Change and %Change in IMCL Feret across the HIIE Bout

	Males	Females	P-Value
SS			
Change	-6.1±36.9	-18.9±27.2	0.78
%Change	1.7±6.1	-2.2±4.6	0.61
Peripheral IMF			
Change	0.6±37.1	-64.9±24.0	0.15
% Change	01.7±4.4	-9.7±3.9	0.19
Central IMF			
Change	29.0±19.7	-38.6±22.3	0.03
% Change	5.2±3.3	-5.6±3.5	0.03
All Areas Average			
Change	-2.3±27.7	-50.2±22.6	0.19
% Change	-1.8±3.6	-7.2±3.5	0.30

Data are expressed as mean±SEM. Comparisons were made using unpaired t-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding IMCL perimeter is found in Figure 4.16 and Table 4.10. IMCL perimeter was significantly affected by sex in the SS ($p=0.004$), peripheral IMF ($p<0.0001$), and total ($p<0.001$) areas, but not in the central IMF ($p=0.28$) area. It was revealed for all of these regions that IMCL perimeter was significantly greater in males. Exercise did not significantly effect perimeter in any region [SS ($p=0.80$), peripheral IMF ($p=0.74$), central IMF ($p=0.98$), or total ($p=0.57$) area]. There was also no significant interaction in the SS ($p=0.96$), peripheral IMF ($p=0.15$) or total

($p=0.54$) area, however there was a sex x time interaction in the central IMF area ($p=0.03$). Post-hoc tests revealed that post-exercise, IMCLperimeter was greater in males than females ($p=0.03$). There was a significant difference in the absolute and % change of IMCL feret in the peripheral IMF ($p=0.01$, $p=0.03$) and central IMF ($p=0.03$, $p=0.04$) whereby the value increased in males but decreased in females. There were no significant differences in absolute change in IMCL feret between the sexes in the SS ($p=0.96$) or total area ($p=0.53$]. There were no significant differences in %change in IMCL feret between the sexes in the SS ($p=0.79$), or total area ($p=0.36$).

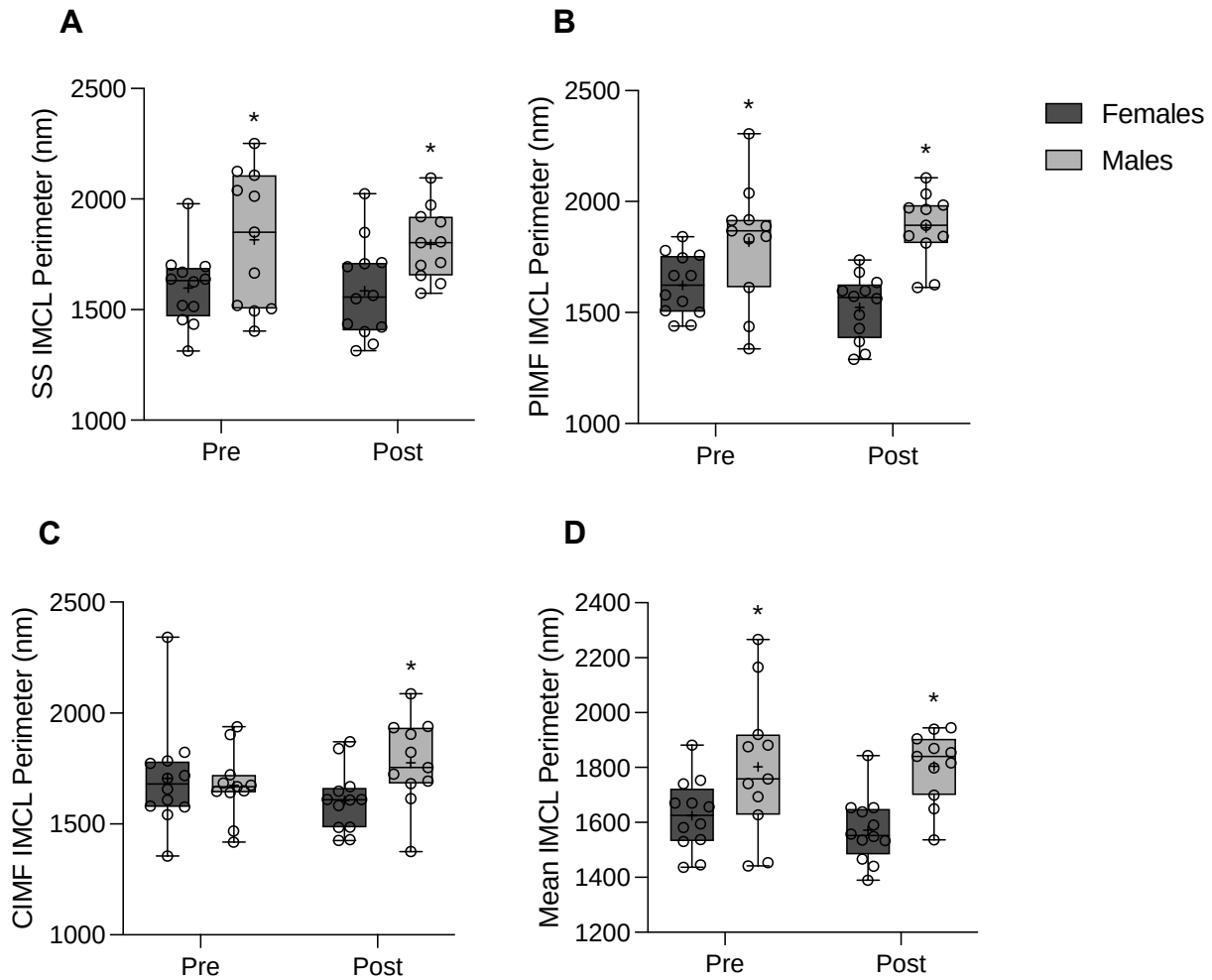


Figure 4.16: Boxplots representing average IMCL perimeter in the A) SS region,

*greater than females, $p=0.004$ B) peripheral IMF, * greater than females, $p<0.001$ C) central IMF, *greater than females post-exercise, $p=0.03$ D) and averaged across all regions, *greater than females $p=0.02$ prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way, mixed model ANOVAs.

Table 4.10. Change and %Change in IMCL Perimeter across the HIIE Bout

	Males	Females	P-Value
SS			
Change	-19.9±111.5	-13.7±69.2	0.96
%Change	1.8±6.4	-0.2±4.1	0.79
Peripheral IMF			
Change	139.0±67.6	-100.7±53.6	0.01
% Change	5.0±2.4	-5.7±3.3	0.03
Central IMF			
Change	101.6±54.5	-100.3±68.7	0.03
% Change	6.4±3.2	-2.4±2.5	0.04
All Areas Average			
Change	2.7±81.4	-53.9±42.6	0.53
% Change	1.9±4.6	-2.9±2.5	0.36

Data are expressed as mean±SEM. Comparisons were made using unpaired t-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding IMCL sphericity is reported in Figure 4.17 and Table 4.11. There was no effect of sex on IMCL sphericity in any region [SS ($p=0.47$), peripheral IMF ($p=0.14$), central IMF ($p=0.59$), total ($p=0.47$) area]. There was no effect of exercise on IMCL sphericity in any region [SS ($p=0.67$), peripheral IMF ($p=0.77$), central IMF ($p=0.83$), total ($p=0.69$) area]. There were no sex x time interactions for IMCL sphericity in any region [SS ($p=0.83$), peripheral IMF ($p=0.10$), central IMF ($p=0.47$), total($p=0.36$) area]. There was a significant difference in absolute and

%change in IMCL sphericity in the peripheral IMF ($p=0.03$ both) whereby the value increased in males but not in females. There were no significant differences in the absolute change in IMCL sphericity between the sexes in any other region of the myocyte [SS ($p=0.90$), central IMF ($p=0.75$) or total area ($p=0.36$)]. There were no significant differences in the %change of IMCL sphericity between the sexes in any area of the myocyte [SS ($p=0.86$), central IMF ($p=0.79$) or total area ($p=0.37$)].

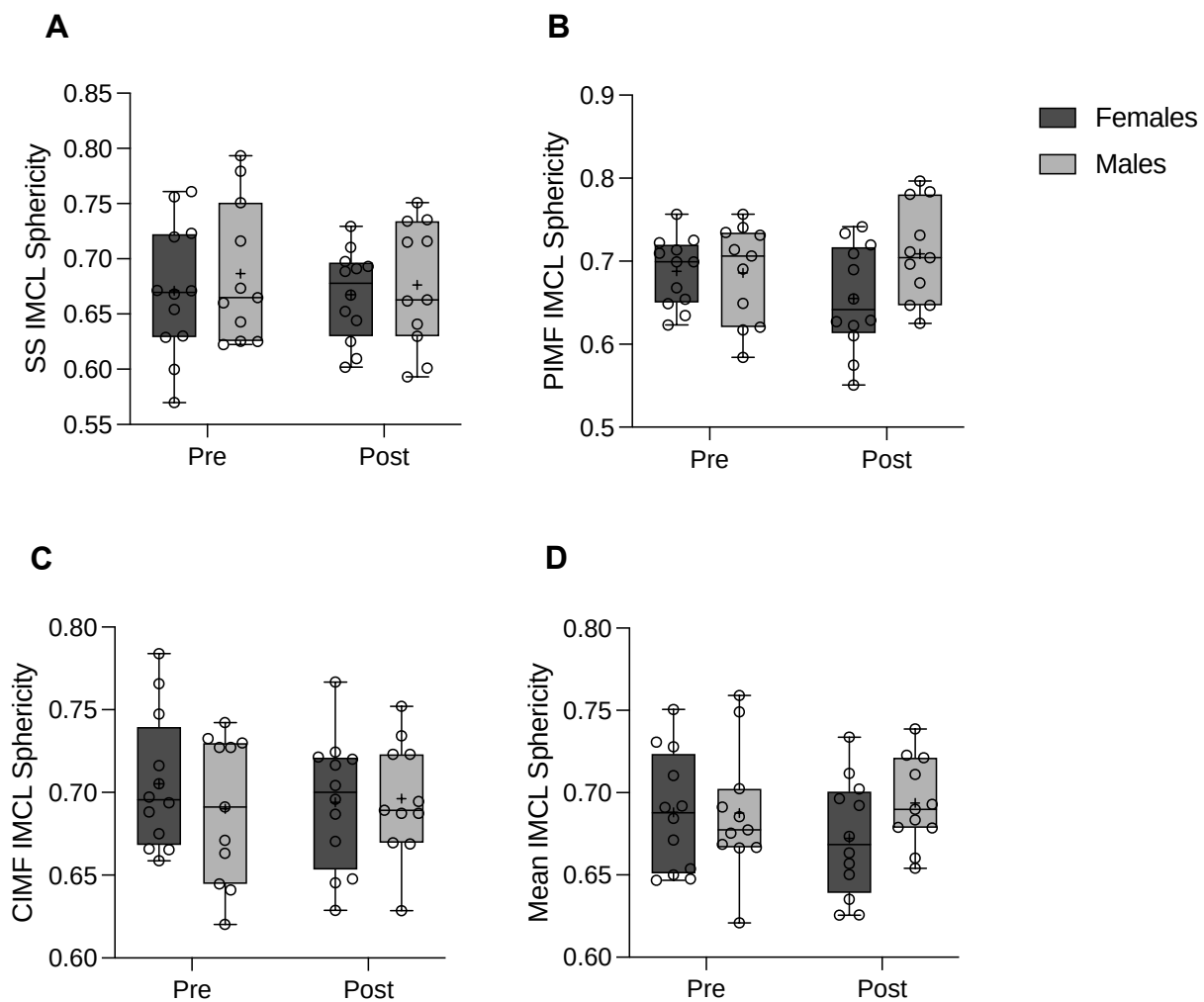


Figure 4.17: Boxplots representing IMCL sphericity in the A) SS region, B) peripheral IMF, C) central IMF and D) averaged across all regions prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of

the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way, mixed model ANOVAs.

Table 4.11. Change and %Change in IMCL Sphericity across the HIIE Bout

	Males	Females	P-Value
SS			
Change	0.007±0.018	0.011±0.018	0.90
%Change	1.5±2.6	2.2±2.8	0.86
Peripheral IMF			
Change	0.038±0.011	-0.033±0.026	0.03
% Change	6.4±1.8	-4.2±3.6	0.03
Central IMF			
Change	-0.004±0.011	-0.011±0.018	0.75
% Change	-0.4±1.6	-1.2±2.5	0.79
All Areas Average			
Change	0.006±0.014	-0.016±0.019	0.36
% Change	1.2±2.0	-1.9±2.7	0.37

Data are expressed as mean±SEM. Comparisons were made using unpaired T-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding the IMCL aspect ratio is reported in Figure 4.18 and Table 4.12. There was no effect of sex on IMCL aspect ratio in any region [SS (p=0.47), peripheral IMF (p=0.52), central IMF (p=0.38), total (p=0.32) area]. There was no effect on exercise on IMCL aspect ratio in any region [SS (p=0.67), peripheral IMF (p=0.32), central IMF (p=0.25) or total(p=0.42) area]. There were no sex x time interactions for IMCL aspect ratio in any region [SS (p=0.84), peripheral IMF (p=0.23), central IMF (p=0.38) or total (p=0.68) area]. There were no significant differences in the absolute change in IMCL aspect ratio between the sexes in any region of the myocyte [SS (p=0.20), peripheral IMF (p=0.23), central IMF (p=0.18) or total area (p=0.82)]. There were no

significant differences in the %change of IMCL aspect ratio between the sexes in any area of the myocyte [SS ($p=0.78$), peripheral IMF ($p=0.08$), central IMF ($p=0.66$) or total area ($p=0.78$)].

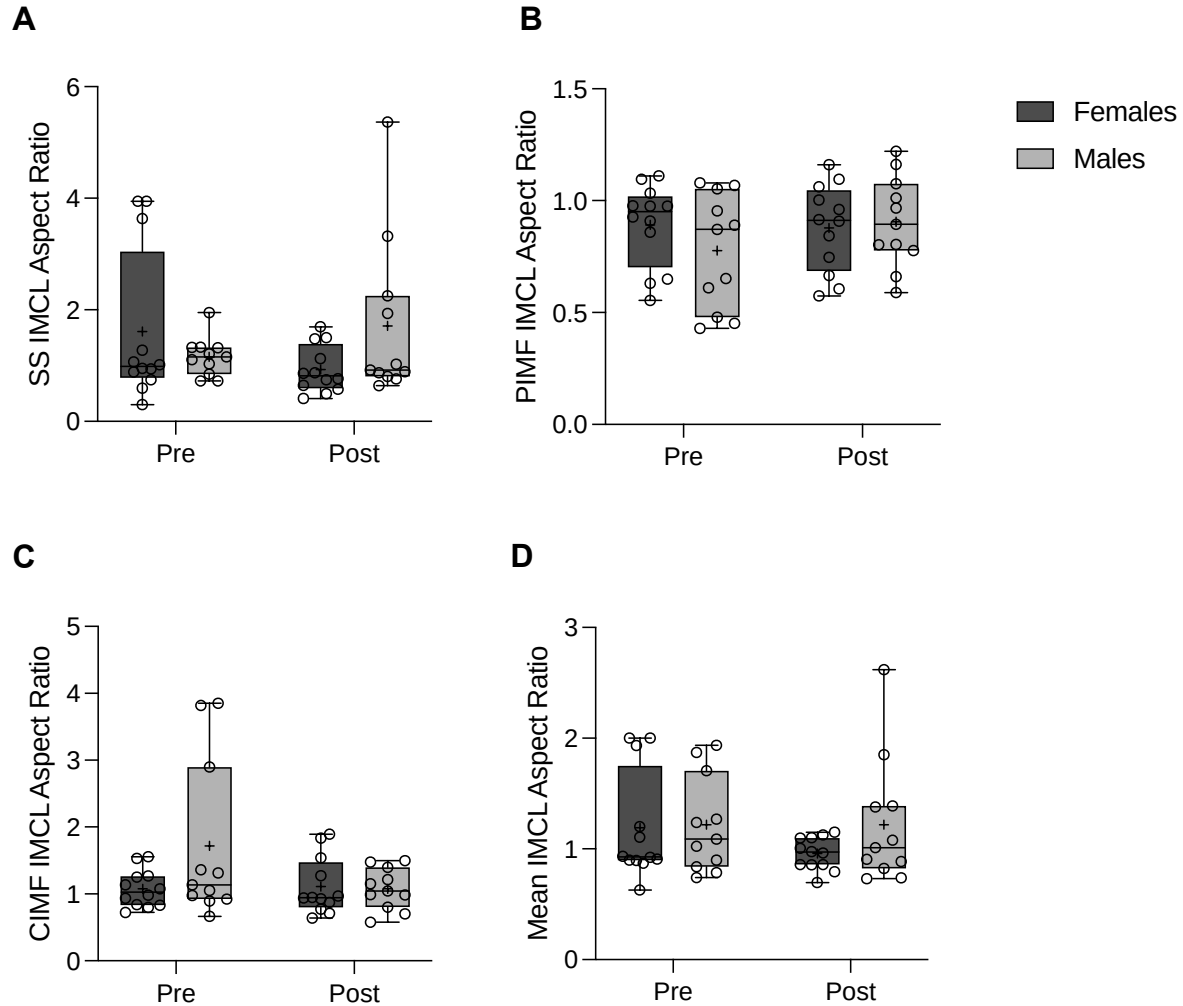


Figure 4.18: Boxplots representing IMCL aspect ratio in the A) SS region, B) peripheral IMF, C) central IMF and D) averaged across all regions prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way mixed model ANOVAs.

Table 4.12. Change and %Change in IMCL Aspect Ratio across the HIIE Bout

	Males	Females	P-Value
SS			
Change	0.15±0.29	-0.42±0.32	0.20
%Change	17.6±21.8	7.9±25.2	0.78
Peripheral IMF			
Change	0.13±0.09	-0.01±0.07	0.23
% Change	30.1±16.7	-3.5±7.8	0.08
Central IMF			
Change	-0.64±0.41	-0.05±0.10	0.18
% Change	-10.7±14.7	-3.2±7.8	0.66
All Areas Average			
Change	-0.19±0.18	-0.14±0.13	0.82
% Change	-7.3±11.4	-3.3±8.5	0.78

Data are expressed as mean±SEM. Comparisons were made using unpaired T-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding the IMCL perimeter-to-area ratio are reported in Figure 4.19 and Table 4.13.

Perimeter-to-area ratio was significantly affected by sex in the SS ($p=0.006$), peripheral IMF region ($p=0.0002$), and total region analyzed ($p=0.0006$) but not any the central IMF ($p=0.18$) region. Post-hoc analyses of the main effect of sex in the all three areas revealed that perimeter to area ratio was significantly greater in females than it was in males. There was no effect of exercise on IMCL perimeter-to-area ratio in any region [SS ($p=0.93$), peripheral IMF ($p=0.62$), central IMF ($p=0.65$), total ($p=0.31$) area]. There was a significant sex x time interaction in the central IMF ($p=0.03$), but not in the SS ($p=0.16$), peripheral IMF ($p=0.11$) or total ($p=0.51$) regions. Post-hoc tests revealed the perimeter-to-area ratio was significantly greater in females than males post-, ($p=0.03$), but not prior to ($p=0.73$) exercise. There was a significant difference

in the absolute and %change in IMCL perimeter to area ratio in the central IMF ($p=0.01$, $p=0.02$) whereby it increased in females and marginally decreased in males. There was no significant difference in absolute change in IMCL perimeter to area ratio in any other region of the myocyte [SS ($p=0.23$), peripheral IMF ($p=0.23$), or total area ($p=0.10$)]. There was no significant difference in %change in IMCL perimeter to area ratio in any other region of the myocyte [SS ($p=0.33$), peripheral IMF ($p=0.55$), or total area ($p=0.17$)].

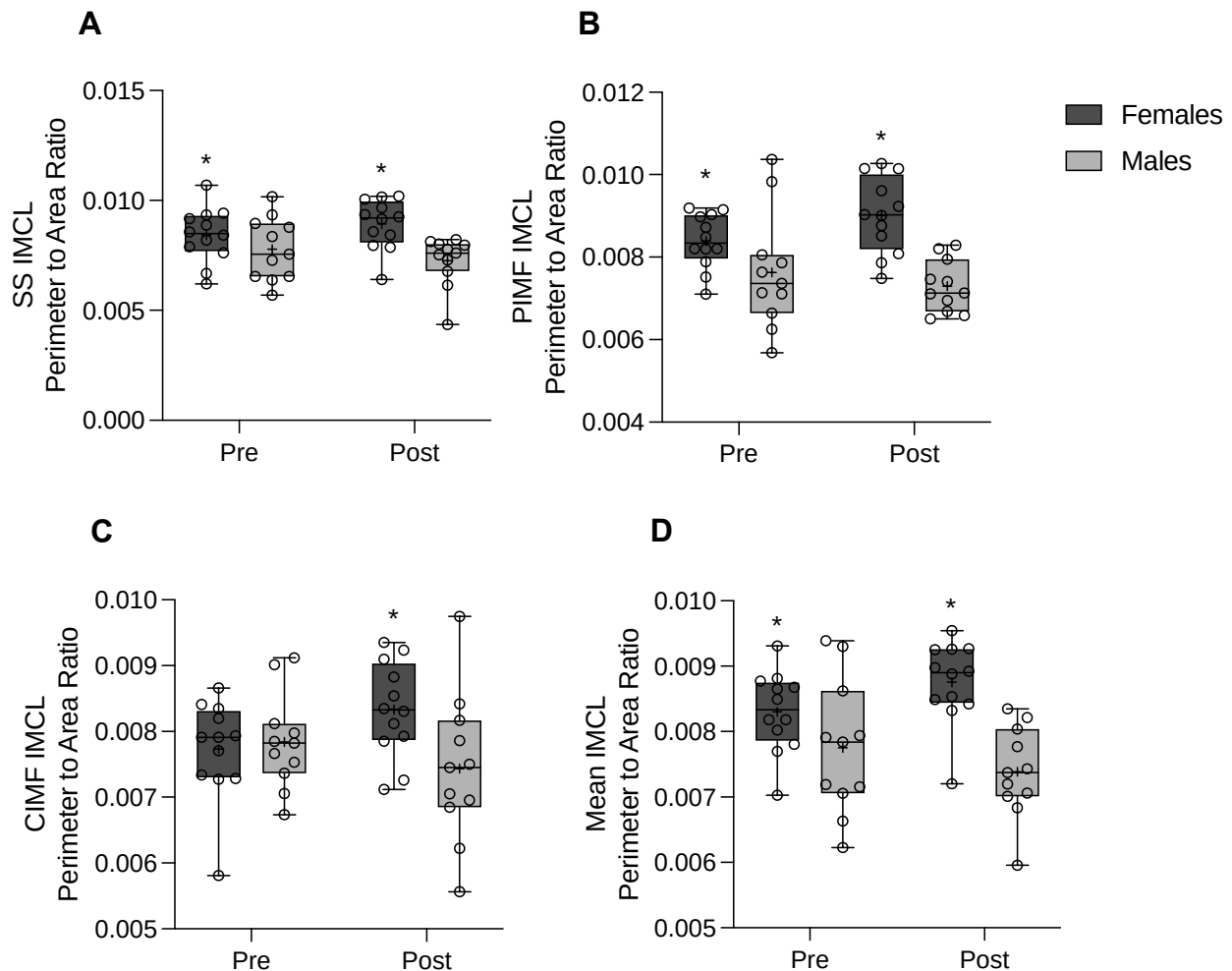


Figure 4.19: Boxplots representing IMCL perimeter-to-area ratio in the A) SS region, *greater than males, $p=0.006$ B) peripheral IMF, * greater than males, $p=0.0002$ C) central IMF, *greater than males post-exercise, $p=0.03$ and D) averaged across all regions, $p=0.0006$ prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile

range (IQR) and extreme values, respectively. Comparisons were made using two-way, mixed model ANOVAs.

Table 4.13. Change and %Change in IMCL Perimeter to Area Ratio across the HIIE Bout

	Males	Females	P-Value
SS			
Change	-0.00040±0.00061	0.00050±0.00043	0.23
%Change	-2.0±8.3	7.9±6.0	0.33
Peripheral IMF			
Change	-0.00002±0.00038	0.00062±0.00036	0.23
% Change	1.4±5.0	5.1±3.6	0.55
Central IMF			
Change	-0.00041±0.00036	0.00076±0.00020	0.01
% Change	-4.7±4.5	7.9±2.1	0.02
All Areas Average			
Change	-0.00044±0.00040	0.00029±0.00017	0.10
% Change	-4.0±5.2	3.6±2.1	0.17

Data are expressed as mean±SEM. Comparisons were made using unpaired T-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Data regarding the percent of IMCL in contact with mitochondria are reported in Figure 4.20 and Table 4.14. There was no effect of sex on the percent of IMCL in contact with mitochondria in the SS (p=0.59), central IMF (p=0.86) or total (p=0.24) area of the myocyte. There was however, a significant effect of sex in the peripheral IMF (p=0.004) where the percent of IMCL in contact with mitochondria was greater in males. There was no significant main effect of exercise on percent of IMCL in contact with mitochondria in any area of the myocyte [SS (p=0.33), peripheral IMF (p=0.16), central IMF (p=0.33), total (p=0.16)]. There was no sex x time interaction for % IMCL touching mitochondria in any region [SS (p=0.16), peripheral IMF

($p=0.36$), central IMF ($p=0.61$) or total ($p=0.13$)]. There were no significant differences in the absolute change in %IMCL touching mitochondria between the sexes in any region of the myocyte [SS ($p=0.34$), peripheral IMF ($p=0.51$), central IMF ($p=0.61$) or total area ($p=0.13$)]. There was a significant %change in %IMCL in contact with mitochondria in the total area ($p=0.02$) whereby it increased in females and slightly decreased in males. There were no significant differences in the %change of %IMCL touching mitochondria between the sexes in any other area of the myocyte [SS ($p=0.33$), peripheral IMF ($p=0.11$), central IMF ($p=0.29$)].

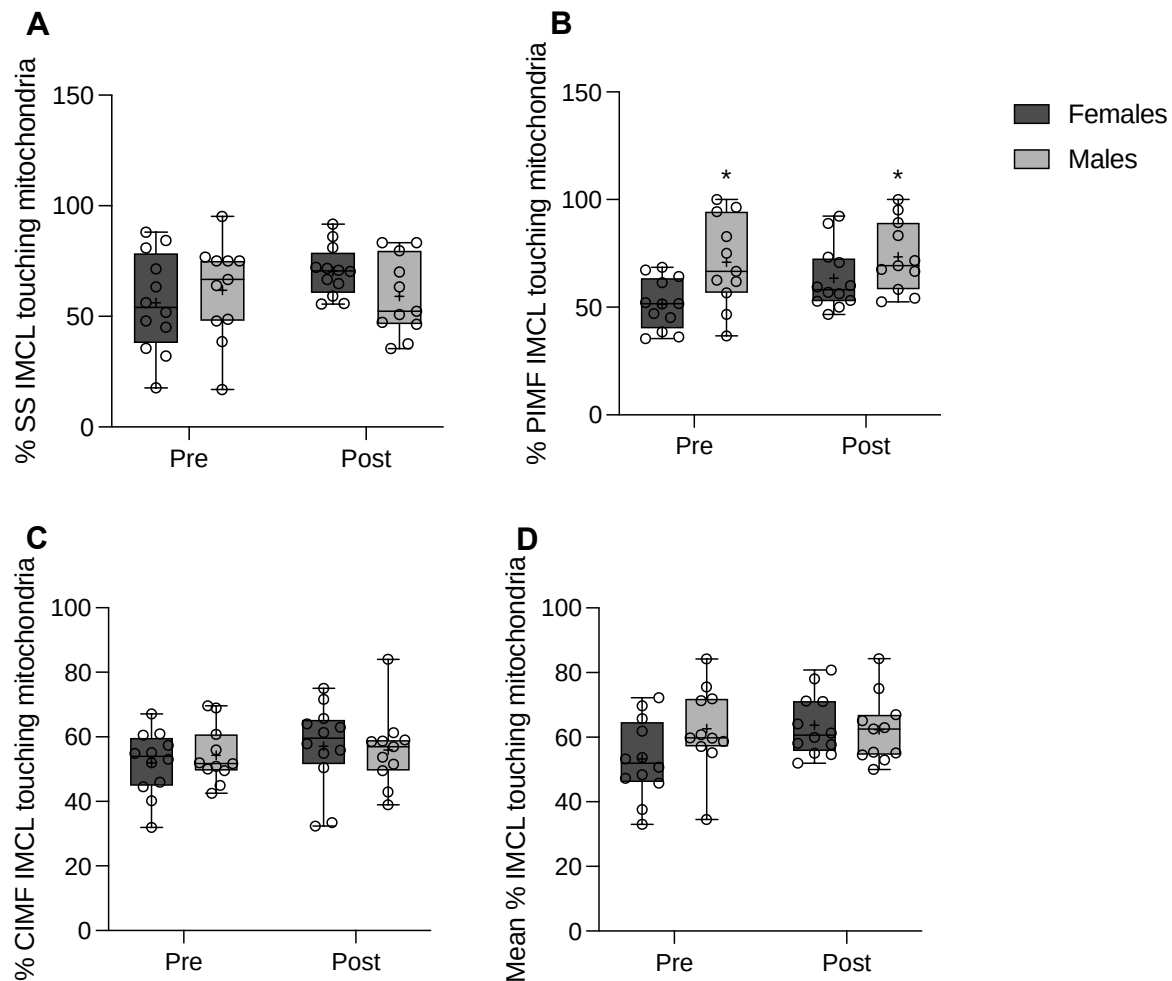


Figure 4.20: Boxplots representing the percent of IMCL in contact with mitochondria in the A) SS region, B) peripheral IMF region, *greater than females, $p=0.004$, C) central IMF region, and D) averaged across all areas prior to and following exercise for males ($n=11$) and females

(n=12). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way, mixed model ANOVAs.

Table 4.14. Change and %Change in % of IMCL in contact with mitochondria across the HIIE Bout

	Males	Females	P-Value
SS			
Change	1.5±10.9	14.3±7.5	0.34
%Change	6.88±19.45	29.93±13.26	0.33
Peripheral IMF			
Change	2.6±9.2	9.2±3.7	0.51
% Change	0.94±9.80	21.37±7.57	0.11
Central IMF			
Change	1.7±4.8	5.2±4.8	0.61
% Change	-0.02±6.95	14.67±10.86	0.29
All Areas Average			
Change	-0.3±5.2	10.4±4.5	0.13
% Change	-3.56±6.55	25.67±9.77	0.02

Data are expressed as mean±SEM. Comparisons were made using unpaired T-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

The average and total length of IMCL-mitochondrial apposition are reported in Figure 4.21 and Table 4.15. We report this in two ways: the average length of contact between a mitochondria and an IMCL droplet in a given region and as the total sum the length of contact between IMCL and mitochondria in a given region. There was a significant main effect of sex on average length of IMCL-mitochondrial apposition for all of the measured areas by which the average length of contact between an IMCL and a mitochondria in the the SS (p=0.03), peripheral IMF (p=0.008), and central IMF (p=0.03), as well as the total area (p=0.03) was greater in males. There was no

significant effect of exercise on the length of IMCL-mitochondrial apposition in any region [SS ($p=0.52$), peripheral IMF ($p=0.13$), and central IMF ($p=0.30$), total ($p=0.38$) area]. There was a significant interaction in the peripheral IMF ($p=0.004$) region that indicated that the length of contact increased in males across exercise ($p=0.01$) and was greater in males than females post- ($p=0.0004$), but not pre- ($p=0.70$) exercise. There were no other significant sex x time interactions for IMCL-mitochondrial apposition length in any region [SS ($p=0.23$), central IMF ($p=0.66$), total ($p=0.64$) areas]. There was a significant difference in the absolute change in average length of IMCL-mitochondria apposition in the peripheral IMF ($p=0.001$), whereby it decreased in females but increased in males. There were no significant differences in the absolute change in average length of IMCL-mitochondria contact between the sexes in any other region of the myocyte [SS ($p=0.42$), central IMF ($p=0.64$) or total area ($p=0.13$)]. There was a significant difference in the %change in average length of IMCL-mitochondria apposition in the peripheral IMF ($p=0.002$) whereby it decreased in females but increased in males. There were no significant differences in the %change of average length of IMCL-mitochondria contact between the sexes in any area of the myocyte [SS ($p=0.56$), central IMF ($p=0.86$) or total area ($p=0.12$)]. Interestingly, there was no significant effect of sex in any of the region for the total length of IMCL-mitochondria contact [SS ($p=0.36$), peripheral IMF ($p=0.27$), central IMF ($p=0.31$), total ($p=0.26$) area]. There was also no significant effect of exercise on the total length of IMCL-mitochondria contact in the SS ($p=0.07$), peripheral IMF ($p=0.48$) or total ($p=0.05$) area. There was a significant main effect of exercise in the central IMF region ($p=0.04$), however post-hoc tests revealed a nonsignificant ($p=0.08$) increase in the total length of contact post-exercise. There was no sex x time interaction for the total length of IMCL-mitochondria contact in any region [SS ($p=0.26$), peripheral IMF ($p=0.10$), central IMF ($p=0.98$) or total ($p=0.78$) area].

There was no significant differences in the absolute change in total length of IMCL-mitochondria contact between the sexes in any region of the myocyte [SS ($p=0.26$), peripheral IMF ($p=0.09$), central IMF ($p=0.98$) or total area ($p=0.78$)]. There were no significant differences in the %change in total length of IMCL-mitochondria contact between the sexes in any area of the myocyte [SS ($p=0.27$), peripheral IMF ($p=0.13$), central IMF ($p=0.52$) or total area ($p=0.97$)].

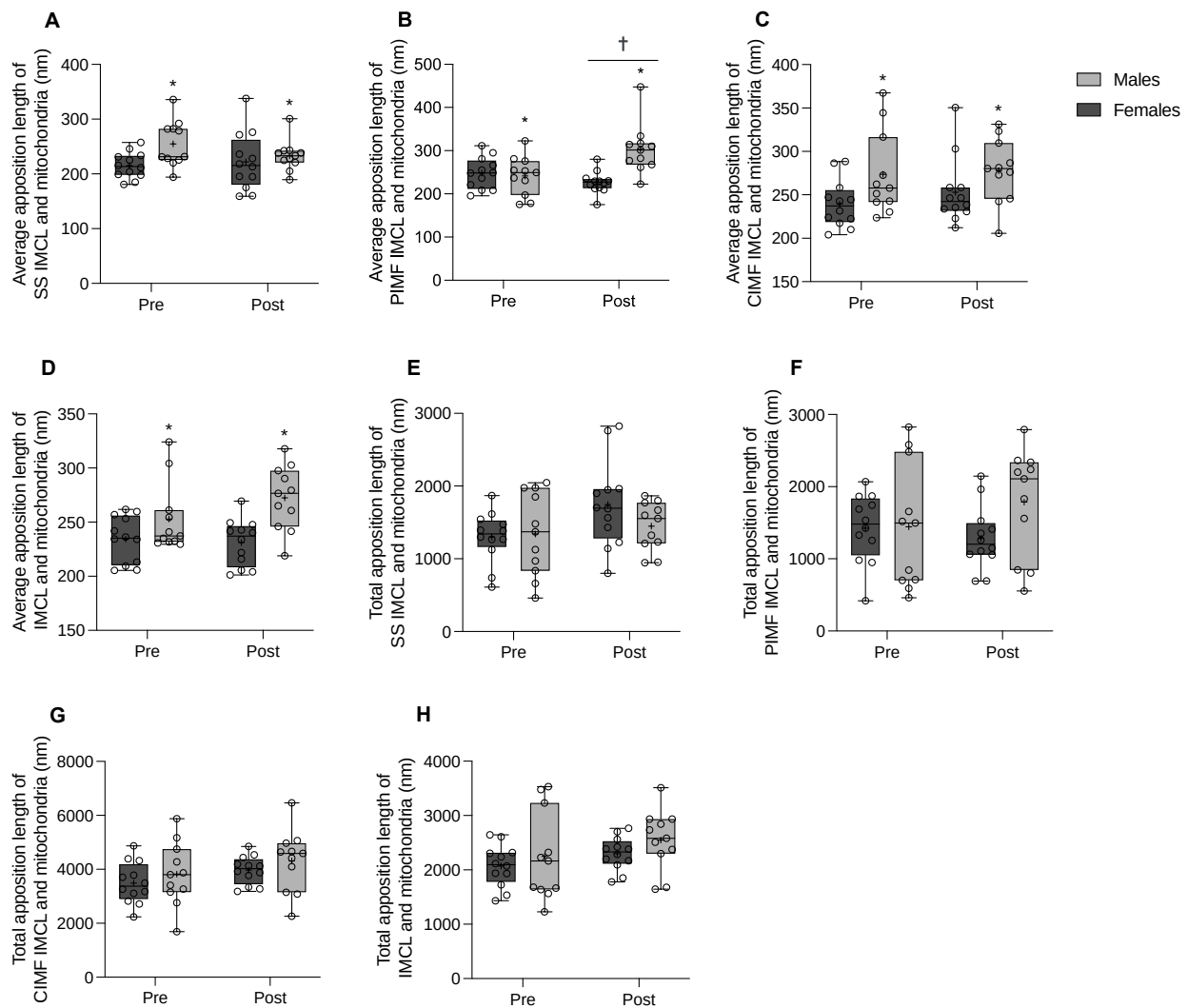


Figure 4.21: Boxplots representing average (panels A-D) and total (panels E-H) length of contact between IMCL and mitochondria in the A) SS region, *greater than females, $p=0.03$ B) peripheral IMF, *greater than females, $p=0.008$, † greater than prior to exercise, $p=0.01$ C) central IMF, *greater than females, $p=0.008$ D) total area, *greater than females, $p=0.008$ E) SS region, *greater than females, $p=0.03$ F) peripheral IMF, *greater than females, $p=0.008$ G) central IMF, *greater than females, $p=0.008$ H) total area, *greater than females, $p=0.008$.

central IMF, *greater than females, $p=0.03$ D) averaged across all regions, *greater than females, $p=0.03$, E) SS region, F) peripheral IMF region, G) central IMF region, and H) averaged across all areas prior to and following exercise for males ($n=11$) and females ($n=12$). The circles represent individual participant values, the cross within the boxplot represents the mean, and the line within the boxplot represents the median. The border of the box and whiskers represent the interquartile range (IQR) and extreme values, respectively. Comparisons were made using two-way, mixed model ANOVAs.

Table 4.15. Change and %Change in length of IMCL-mitochondria apposition in the HIIE Bout

	Males	Females	P-Value
Average Length of Contact			
SS			
Change	-21.5±15.4	-5.0±13.2	0.42
%Change	-6.3±5.5	-1.6±5.7	0.56
Peripheral IMF			
Change	61.9±21.9	-31.2±11.2	0.001
% Change	29.9±10.5	-11.1±3.9	0.002
Central IMF			
Change	4.1±16.8	13.3±10.5	0.64
% Change	3.8±6.3	2.6±2.8	0.86
All Areas Average			
Change	19.5±12.2	-3.1±8.3	0.13
% Change	8.9±4.9	-0.6±3.6	0.12
Total Length of Contact			
SS			
Change	106.0±204.3	446.7±207.8	0.26
%Change	16.3±18.8	46.7±19.1	0.27
Peripheral IMF			
Change	341.9±230.9	-142.7±162.9	0.09
% Change	32.0±24.2	-9.7±12.5	0.13
Central IMF			
Change	489.7±411.3	476.8±187.6	0.98
% Change	9.4±10.4	17.3±6.7	0.52
All Areas Average			
Change	300.8±235.1	226.8±123.3	0.78
% Change	14.6±10.8	14.2±7.0	0.97

Data are expressed as mean±SEM. Comparisons were made using unpaired t-tests. SS: Subsarcolemma, IMF: intermyofibrillar.

Chapter Five: Discussion

5.0 Overall Summary

This study aimed to characterize sex differences in IMCL storage, localization, and IMCL-mitochondria interaction during an acute bout of HIIE. Our study revealed subcellular region dependent sex differences in IMCL size, feret, perimeter, and perimeter-to-area ratio. We found no effect of sex or exercise for sphericity, aspect ratio or IMCL area density. With respect to IMCL-mitochondria interaction, we found subcellular region dependent sex differences in length of IMCL-mitochondria apposition and the percentage of mitochondria in contact with IMCL. We also examined whether sex influenced metabolism during the exercise bout. While we observed similar RER during exercise between the sexes, females had lower rates of CHO oxidation and a lesser increase of blood lactate than males. Importantly, this occurred despite females exercising at a higher intensity than males during the acute exercise bout, as evidenced by achieving a higher percentage of their target heart rate and percentage of VO₂peak during high intensity intervals. We found no difference in the content of lipolytic (ATGL, PLIN3), IMCL turnover (PLIN2, PLIN5), lipid metabolism (CPT1, SCHAD) or aerobic metabolism (COX I-V, CS) proteins between males and females. Collectively, our findings suggest that IMCL do not contribute substantially to aerobic metabolism during an acute bout of HIIE, but females rely to a lesser reliance on anaerobic glycolysis.

5.1 Sex-based differences in lipid storage

5.1.1 Sex-based differences in IMCL size, number and area density (%)

Most studies characterizing IMCL storage using TEM have reported IMCL area density, IMCL size and IMCL number across the entire myocyte^{17,21}. Although IMCL content has been

associated with insulin resistance in obese individuals^{71,148}, endurance athletes also store greater IMCL¹⁴⁸ and thus the nature of this relationship is more complicated than content alone. Specifically, the accumulation of larger IMCL localized in the SS region is suggested to be associated with IR^{69,79}. Indeed, in endurance trained individuals, the greater IMCL content is owed to a greater number of smaller IMCL in the IMF^{45,72,79}. In the current study, we investigated IMCL storage characteristics by subcellular region in an effort to address whether sex influences how IMCL are stored within the myocyte. We report a significant main effect of sex on IMCL size, by which IMCL were larger in males in all regions of the myocyte. Additionally, we report that females had a greater number of IMCL per unit of area in the SS, peripheral IMF, and total area, but not in the central IMF region. Interestingly, we do not report any differences between the sexes in IMCL area density % in any subcellular region. Thus, males had a lesser number of larger IMCL in all regions but the central IMF, which would tend to be in line with a phenotype associated with IR. However, this parallel is not entirely aligned as males did not also have a greater IMCL content than females. Thus our findings highlight that for the most part differences in IMCL storage between males and females are consistent across the myocyte.

Previous work has found that females have a greater IMCL area density as a result of storage of a greater number of IMCL, with IMCL size not differing between the sexes^{17,27}. Thus our finding that IMCL area density did not differ between males and females is likely due to the fact that while our females stored a greater number of IMCL, IMCL size was greater in males. Since IMCL area density is a function of IMCL size and number, our findings highlight that for a given IMCL content, IMCL is stored differently in males and females. Our lack of sex difference in IMCL area density is difficult to reconcile given that Devries and colleagues' participants

were matched for VO₂peak relative to FFM and their fitness indices were nearly identical to ours¹⁷. However, given that it has also been shown in the literature that IMCL content is 3-fold greater in type I muscle fibers⁵⁸, the lack of difference in IMCL area density in the current trial may be attributed to similarities in fibre type profiles between the sexes. To our knowledge, neither study that has reported greater IMCL content in females^{13,17} reported fibre type profiles. Thus, not accounting for these differences could contribute to our differential reporting.

Interestingly, our finding that IMCL size is greater in males contrasts what has been found before by Devries and colleagues¹⁷, whereby they noted no difference in IMCL size between the sexes. We find it difficult to reconcile these findings given the previously mentioned similarities in matching and participant profiles¹⁷. However, this may again be due to the fact that fibre type did not differ between males and females in the current study and thus a further examination of sex-based differences in IMCL storage with consideration of muscle fibre type is required. Interestingly, the sex difference in IMCL size found in the current trial could have implications for improving our understanding as to why males have a greater risk of developing type 2 diabetes than females^{82–84,149}. Storage of larger lipid droplets is associated with insulin resistance, and thus the fact that our young healthy males stored IMCL as larger lipid droplets could point to a physiological reason as to why insulin resistance is greater in males than females^{149,150}. However, data from this cohort of participants published previously did not find a difference in insulin sensitivity between males and females¹⁴⁰. Examination of sex-based differences in IMCL storage characteristics across the spectrum of insulin sensitivity would be interesting to determine whether differences in IMCL size between the sexes contributes to differences in the development of insulin resistance and type 2 diabetes between males and females.

We also examined the effect of sex on IMCL-mitochondria apposition, decomposing the concept into the average length of contact, the sum of all lengths of contact for a given region, and % of IMCL in contact with mitochondria. While average apposition length was greater in males in all regions, there was no difference due to sex for total length of apposition. Thus, while individual apposition length was greater in males, this did not translate to an overall increase in measurable contact between IMCL and mitochondria. To date, we are aware of only one other group¹⁴⁷, who has assessed length of IMCL-mitochondrial apposition. They reported similar absolute lengths of contact (~140nm-430nm). It should be noted however, that their participants were sedentary males between 40-65 years of age, and 1/3 were diagnosed diabetics¹⁴⁷. Going forward it would be interesting to further examine how age, training/de-training and extent of insulin resistance influences IMCL-mitochondrial apposition length in an attempt to determine the importance of this measure. However, as mentioned previously,^{28,148} it has been suggested that greater IMCL-mitochondria interaction could be indicative of enhanced IMCL breakdown and flux into the mitochondria for oxidation¹⁴⁷. Conversely, it has also been posited that this same interaction is a sign of IMCL deposition and that the involved mitochondria were involved in providing energy for the synthesis of TAG¹⁵¹. Similar to our study, a previous study has found no difference between the sexes for % of IMCL in contact with mitochondria at baseline¹⁷. Although we noted a greater average length of contact, our findings largely corroborate these results as we also noted that the total length of contact was not different between the sexes. Thus, together, our results suggest that while there may be slight differences in IMCL-mitochondrial apposition between the sexes, it is likely that at baseline males and females have similar capacities for IMCL breakdown *or* synthesis, as the nature of this interaction still remains to be elucidated.

5.1.2 Sex-based differences in IMCL morphology

Excitingly, we also examined novel morphological markers such as feret, P:A ratio, sphericity and aspect ratio, which have only been reported once by de Almeida and colleagues¹⁴⁷, and never in females. IMCL feret and perimeter were greater in males, whereas the perimeter-to-area ratio was greater in females, and there were no differences in IMCL sphericity or aspect ratio between the sexes. Specifically, we found that IMCL feret in males was greater in the peripheral IMF and total area with a tendency to be greater in the SS. Given our findings that IMCL area was larger in males, a wider feret is not surprising as feret is defined as the longest distance across the lipid droplet. A wider feret may suggest a more oblong IMCL; however, we did not find a difference in IMCL sphericity between the sexes. Because of this, a wider feret in males might be more indicative of a larger lipid droplet of similar circularity. This hypothesis is further supported by the finding that while the perimeter was greater in males, perimeter to area ratio was greater in females in the SS, peripheral IMF and averaged across all areas irrespective of exercise. Thus, the greater perimeter in males is not necessarily suggestive of a lipolytic advantage in males as a longer perimeter would be necessary to enclose a larger area. Since a circle has the smallest perimeter for the greatest area, storing a larger circular lipid droplet instead of an oblong or irregularly shaped lipid droplet would be less optimal for contact with mitochondria. Our data are suggestive that compared with healthy, equally trained females, males store lipid in a manner more in line with that seen in insulin resistance, as de Almeida and colleagues also reported a significantly greater SS IMCL perimeter length in obese and T2D males compared with lean males¹⁴⁷. Interestingly, de Almeida and colleagues reported no difference in IMCL perimeter in the IMF region between lean, obese and T2Ds individuals¹⁴⁷. In the current trial we separated the IMF into peripheral and central IMF regions as previous work

has reported greater IMCL utilization in the peripheral region of the myocyte during exercise¹⁵². Here we found that the perimeter was markedly different between the sexes in the peripheral IMF, but less so in the central. Taken together, the greater feret, perimeter and lipid droplet area in males in the peripheral IMF suggests that these lipid droplets have a lesser surface area for contact with mitochondria and metabolic enzymes, which may limit lipid turnover.^{151,152}

Interestingly, while the sex difference in IMCL size and average apposition length was found in all regions of the myocyte, the sex difference in the proportion of IMCL in contact with mitochondria, IMCL feret and IMCL P:A ratio were only different in the peripheral IMF and perimeter was different only the SS and IMF region. The differential effect of region might be an indicator that characteristics such as size and apposition length may be more determined by sex, whereas differences in proportional contact, feret perimeter and P:A ratio may be linked to environments specific to each region, such as mitochondrial abundance or shape. Indeed, there are distinct differences between SS and IMF mitochondria¹⁵³, both in morphological characteristics and posited uses, thus the IMCL interacting with it in each region may also be unique.

5.2 Fuel utilization during an acute bout of HIIE in males and females

5.2.1 Females worked at a higher relative exercise intensity and yet used a similar amount of fat as males, and less CHO than males

The HIIE bout used in this trial was used as it is commonly used in HIIE training studies^{154–158}. In these trials individuals train at a low intensity for the warm up and cool down and either rest or work at a low intensity in between each high intensity interval. While exercise intensity for the high intensity intervals is always prescribed as a relative workrate (i.e. 90%

HRmax), the intensity for the warm up, cool down and between intervals has been prescribed at a fixed rate (i.e. 50W) irrespective of sex. As such, the relative intensity during the warm up, cool down and between intervals is higher in females compared with males. In the current trial this translated to females cycling at a greater intensity over the exercise bout, evidenced by females achieving a higher % target heart rate than males (females: $101 \pm 1\%$, males: $93 \pm 2\%$) as well as a working at a higher average %VO₂peak (females: $69 \pm 3\%$, males: $55 \pm 3\%$).

Exercise intensity is well evidenced to affect substrate utilization^{7,33,158}, with CHO oxidation increasing with increasing exercise intensity^{7,33,159}. Due to this increased exercise intensity, it would be conceivable that females would rely to a greater extent on CHO, which would be evidenced by a higher exercise RER, as well as a greater increase in lactate. Interestingly, this was not the case in the present study. Indeed, we noted a greater increase in blood lactate in males, alongside a similar exercise RER between the sexes. While we found no effect of sex on fat oxidation during exercise, males had a higher rate of CHO oxidation and oxidized more CHO both in absolute terms and relative to BW than females. Thus, despite working at a greater intensity, females had a lesser accumulation of lactate, a lesser reliance on CHO, and a similar utilization of fat as males. Together these findings are in line with previous work that has shown that females have a lower RER and rely to a lesser extent on CHO sources during exercise at a given intensity^{11,14,86,128,160–163}. However, it should be noted that these findings may be less reliable since, while they have been used to calculate fuel utilization during interval exercise previously¹⁶⁴ given the short duration of each interval (1 min) it is highly unlikely that steady state was achieved during any interval.

The greater increase of blood lactate in males may mechanistically be cause and consequence of their greater reliance on CHO. As mentioned previously, lactate is posited to

limit fat oxidation in multiple ways; through antilipolytic activity on a GPCR upstream of adenylate cyclase³⁸⁻⁴⁰, through inhibiting the release FFA following lipolysis³⁷, and by limiting transport of LCFA into the mitochondria by inhibition of CPT1^{46,165}. It has been reported that at the outset of exercise females are quicker to initiate aerobic metabolism than males^{166,167}. Thus, it is possible that if males initially relied on anaerobic glycolysis more after warm-up, they would thereby release more lactate as a by-product of glycolytic flux^{38,39,41}. This would further limit fat oxidation and cyclically increase their reliance on CHO as a substrate. It would be valuable to investigate which, if any of these mechanisms were underlying the sex differences we noted. Thus, future studies could benefit from assessment of FFA rate of appearance during HIIE, as well as activity of CPT1.

In the current trial we found a minimal contribution of fat to energy production during HIIE. Specifically we found that the relative contribution of fat to energy production decreased during exercise, falling as low as 3%. Furthermore, we estimate that only 0.5-0.7 grams of fat were oxidized per kilogram of FFM for males and females respectively during the exercise bout. In females, this is most likely attributed to the average intensity of the HIIE bout (69% VO₂max), which is beyond the intensity where maximal fat oxidation occurs (~62-64% VO₂max). A significant contribution of CHO and minimal fat utilization has been reported by previous SIE studies^{36,59,60}. If the high and rest intervals of these protocols are averaged, the intensity of the SIE bouts were 70%VO₂peak^{36,59,60}, which is in line with the average %VO₂peak that our female participants were cycling at. Thus, our findings are in line with previous trials that when exercise intensity is at or greater than 70% VO₂peak, fat does not contribute substantially to energy production during the exercise bout. It is harder to reconcile the lack of fat oxidation during the acute exercise bout for males, as at roughly 55%VO₂peak,

our males were exercising near the average intensity reported by Essen and colleagues⁵⁸, who reported that fat contributed 40% of energy production during the HIIE bout. Importantly, their exercise bout lasted 60 minutes compared to ours, which only lasted 30 minutes. Additionally, their study did not mention controlling for the fasted vs fed state⁵⁸, whereas all our participants arrived fasted. As there is a greater reliance on fats as substrates during fasted exercise¹⁶⁸, it compounds the difficulty we have to explain the disparity between our findings. However, differences in exercise modalities are also important to consider, as half of our exercise bout was spent at 90%HRmax, which is beyond the lactate threshold¹⁶⁹. Taking into consideration the previously mentioned mechanisms by which lactate may inhibit lipolysis, FFA release and/or entry of FA into the mitochondria, the greater increase of blood lactate observed in males may have contributed to their reduced use of fat despite still cycling at a lower intensity. Indeed, although there was an initial jump to 4mmol/L, Essen and colleagues reported blood lactate values of roughly 2.5mmol/L and below during their 60 minute MICE bout at 55%VO₂max⁵⁸. Moreover, we need to consider the maximal lactate steady state (MLSS), a term used by clinicians to define the maximal intensity of continuous exercise by which lactate remains steady¹⁷⁰. Because lactate concentrations at MLSS have plateaued and are not increasing, the exercise intensity at MLSS is below the lactate threshold¹⁷⁰. At the exercise intensity corresponding to MLSS, lactate concentrations range from 1.5 to just below 7mM, and average around 3.7mM¹⁷⁰⁻¹⁷². Our males had an average blood lactate concentration of roughly 5mM, thus above the average blood lactate at MLSS as well, and were likely much more susceptible to fat oxidation decreases due to lactate accumulation. It is notable that Essen⁵⁸ assessed the rate of lipid oxidation by infusion of oleic acid and periodic draws of blood, then calculating fractional uptake and turnover, which is a much more direct method compared with calculations from RER

values, especially during HIIE. Thus, further investigation into sex differences in fat metabolism and sources of lipid are still required, keeping in mind relative intensity of rest as well as method of assessment.

5.2.2 IMCL is not used during an acute bout of HIIE

The findings from the current trial suggest that it is unlikely that IMCL was a significant contributor to energy production during the acute HIIE bout in either males or females. Previous studies^{17,18,152,173} reported IMCL utilization as evidenced by a decrease in IMCL area density after 60-90 minutes of cycling near 60%VO₂peak. We contrast this report as we found no change in IMCL area density during HIIE in either males or females. We are not necessarily surprised that we did not see a decrease in IMCL during our 30 minute bout of HIIE as it may have been too short of a duration for IMCL contribution to be significant, as classical reports of IMCL utilization have come after either 60 minutes of exhaustive exercise^{147,174}, or 60-90 minutes of moderate intensity continuous exercise^{13,17,87}. Interestingly, reports by Romijn and colleagues show an 11% and 17% contribution of IMCL to energy expenditure after 30 minutes cycling at 85%VO₂peak^{34,86} which was nonsignificant between the sexes. Again, however, we suggest that the interval nature and/or greater intensity of our high intensity intervals that resulted in a substantial increase in blood lactate concentration may have limited fat oxidation. Indeed, while blood lactate was high in their individuals at 85%VO₂max, the values were noted to be steady and in fact decreased from 7.9 to 7.3mM⁸⁶ suggesting they were in MLSS¹⁷⁰ while our participants were not. Moreover, all participants in both studies were endurance trained athletes^{34,86} whereas ours were not, and thus their participants may be more able to sustain work at higher intensities. While IMCL may not have been a significant observable contributor during HIIE, it has been well established that acute exercise elicits a state of elevated post-exercise

oxygen consumption (EPOC)^{175,176}. During this time, it has also been shown that TAG cycling and a shift toward utilization of fats may contribute to energy production during this prolonged state¹⁷⁵. Moreover, it has also been reported that lipid utilization and EPOC are increased in an intensity-dependent manner whereby vigorous exercise and SIE elicited the greatest increases¹⁷⁶. Thus, deeper investigation into the contributions of IMCL to substrate utilization during, but especially *after* an acute bout of HIIE may be provide valuable insights into the role of fat and IMCL to energy production to exercise and post-exercise energy production. To the best of our knowledge, IMCL utilization has not been characterized across an acute bout of HIIE in any other study, thus more work in this area is required. Overall, our findings suggest that IMCL do not contribute substantially to energy production during an acute bout of HIIE in males and females, which is in line with our finding of minimal overall fat oxidation during the HIIE bout.

5.2.3 No difference in IMCL utilization or IMCL-mitochondrial apposition during HIIE between males and females

Since IMCL did not decline during the HIIE bout we obviously did not find an effect of sex on IMCL utilization. Previous work has yielded conflicting results with studies showing males used more¹⁶, less^{13,19,87} or similar amounts of IMCL^{17,18} during moderate-intensity aerobic exercise. These discordant findings may be due to multiple factors from the sensitivity of analytical methods to matching of participants. Indeed, reports of a lesser utilization of IMCL in males have used the biochemical extraction method^{13,19,87}, which is susceptible to contamination by EMCL. While Zehnder and colleagues' utilized TEM¹⁶, their report of a greater utilization of IMCL in males contrasts findings found in other TEM¹⁷ and H-MRS¹⁸ studies. This can be reconciled as improper matching between the sexes; the males the Zehnder study were significantly more trained than the females¹⁶. Thus, it was not a sex effect but rather a training

effect that males used more IMCL in their study. When participants are properly matched for training status and gold standard assessments such as TEM^{17,18} and H-MRS¹⁸ are used, studies report similar utilization of IMCL between the sexes. The findings of the current trial once again suggest that sex does not influence IMCL utilization during exercise when males and females are properly matched for aerobic fitness.

We additionally investigated whether sex influenced the effects of HIIE on IMCL-mitochondrial apposition. Previous work has reported that IMCL-mitochondrial apposition increases in females, but not males, following a 90-minute bout of moderate intensity aerobic exercise¹⁷. However, de Almeida and colleagues found that IMCL-mitochondrial apposition increased in males following 60 minutes of cycling at 62% or 72% of VO₂peak¹⁴⁷. Here we report a greater % IMCL in contact with mitochondria in the peripheral IMF in males with no difference due to exercise. Interesting, however is that the % change in IMCL-mitochondrial apposition in the total muscle was different between the sexes (females: +5.67±9.77%, males: -3.56±6.55%). The finding of a greater change in the % IMCL in contact with mitochondria in females is in line with the aforementioned findings from Devries and colleagues¹⁷. However, we also found that the change in the average length of contact between IMCL and mitochondrial in the peripheral IMF also differed between the sexes (females: -31.2±11.2%, males +61.9±21.9%). Furthermore, the change in total length of contact in the peripheral IMF region also tended to differ between males and females (p=0.09, females: -142.7±162.9%, males + 341.9±230.9%). These differential findings highlight the importance of characterizing not just the % of IMCL in contact with mitochondria, but also the length of apposition. However, further work determining the physiological meaning and importance of IMCL-mitochondrial apposition is required.

5.2.4 The lack of difference in whole body fat oxidation and IMCL utilization may be due to the lack of difference in content of proteins related to lipid and oxidative metabolism and/or muscle fibre type.

We examined the content of PLIN 3 PLIN 5, PLIN 2 and ATGL as indicators of IMCL turnover and lipolysis, respectively and found no differences between the sexes. Our finding that PLIN 2 did not differ between the sexes aligns with another report of a lack of sex differences in healthy individuals between the ages of 18-30 years¹⁷⁷. However, our finding that PLIN 2, PLIN 3 and PLIN 5 did not differ between the sexes is in contrast with previous work that reported that the content of these proteins were greater in females than males^{17,68,178}. However, our findings of a lack of difference in these proteins are in line with our finding that sex did not influence IMCL utilization, whole body fat oxidation or the % contribution of fat to TEE during HIIE. Our discordance with the Peters report is difficult to explain as we both matched between the sexes by VO₂peak relative to FFM⁶⁸ and tested in the same menstrual phase. We suggest that our differential findings may be due to differences in western blotting methodologies used in the two studies as Peters et al used the muscle pellet⁶⁸, whereas we used the muscle supernatant. Thus future work may need to examine whether PLIN protein content differs in the supernatant vs the pellet and what implications this has for sex-based differences in metabolism. Our findings also differ from those of Bosma and colleagues; however, they did not report matching males and females for training status, which is known to affect content of such proteins¹⁷⁸, and thus may be the reason for the discordant findings.

Our findings align with two other papers that reported no difference in ATGL protein content between the sexes^{179,180}. However, protein content does not necessarily reflect enzyme activity. Indeed, Moro and colleagues reported a greater activity of ATGL in females compared

with males¹⁰⁸. Unfortunately, we did not assess ATGL activity in the current trial and thus it is possible that ATGL activity was greater in females during HIIE despite similar protein content. However, as there was no difference in IMCL breakdown in the current trial, even if ATGL activity was greater in females it did not translate to greater IMCL utilization. Importantly, it has been shown that ATGL expression in adipose cells is upregulated by 17- β estradiol's effect on estrogen receptor alpha¹⁸¹ but not in cells treated with lipopolysaccharide as a model of inflammation. As HIIE has been shown to acutely increase inflammation¹⁸², it would be valuable for future studies to assess ATGL expression, content, and activity to improve our knowledge on the regulation of TAG mobilisation and lipid metabolism during an acute bout of HIIE.

The current trial measured content of CPT1 and SCHAD as indicators of lipid oxidation and found no differences between the sexes. Our findings are in line with previous reports that while females have greater expression of CPT1, this did not translate to a greater protein content or activity⁴⁷. In the current trial we noted a greater increase in blood lactate in males compared with females, which might suggest that CPT1 activity was greater in females as lactate inhibits CPT1 activity. If true, this could underpin the greater reliance on CHO by males in the current trial. While we did not assess CPT1 activity, it would be worthwhile for future studies to do so.

Similarly, our findings align with another study showing protein content of SCHAD to be equal between the sexes¹⁰⁷. However, this same study noted greater content of TFPalpha, VLCAD, and MCAD in females¹⁰⁷. While short to medium chain fatty acids can pass into the mitochondria via passive diffusion, longer chain fatty acids are transported via the carnitine shuttle¹⁸³. Thus, the finding that the content of proteins related to medium to very long chain fatty acid metabolism are in higher abundance in females while SCHAD was not may still speak to a sex difference in capacity to transport FA into the mitochondria for beta oxidation¹⁸³. To

elucidate this, future studies should investigate the activity of TFPalpha, VLCAD, MCAD and CPT1 during exercise alongside careful examination of IMCL breakdown and utilization to determine how sex influences lipid metabolism during exercise.

We also measured the content of Complex I-V and CS as indicators of oxidative phosphorylation and report no sex differences. Other studies have reported similar OXPHOS protein content between the sexes in young, healthy adult males and females^{179,184}.; however, in one of those studies participants were only matched for age and BMI.¹⁷⁷ In this study, while reported habitual activity was similar between the sexes, it is unclear whether they were matched for aerobic fitness¹⁷⁹. In the other study, all participants were reportedly sedentary, yet again, there was no mention of specific testing and matching for aerobic fitness. Since an increase in mitochondrial content and function is one of the main adaptations to endurance training, the lack of matching of males and females for training brings into question the validity of findings from any study that does not properly match males and females for aerobic fitness. Furthermore, a recent meta-analysis that included results from 3 studies reported that Complex IV content alone tended to be greater in males than females¹⁸⁵. Although the meta-analysis excluded studies with uneven sex ratios, they did not account for matching by fitness¹⁸⁵. Indeed, none of the three reported intentional matching for physical fitness, at best comparing reported physical activity^{186–188}. Thus, overall it appears that sex has a minor impact on the content of electron transport chain proteins, but further investigation with consideration of matching for fitness should be done. In the current trial we did not assess electron transport chain activity; however, findings from other trials have found that sex may affect OXPHOS complex activity differently, where two studies showed males having a greater activity of Complex II^{189,190}, and females of Complex IV¹⁹¹. However, again the applicability of these findings are hampered by the fact that

males and females were not properly matched for aerobic fitness. Our finding that CS protein content was similar between the sexes aligns with another study also reporting no effect of sex on CS content¹⁹². However, their study did not report matching, only a lack of difference of VO2peak relative to BW¹⁹². It is our opinion that participants should have been matched due to VO2 peak relative to FFM as is recommended as the gold-standard for sex comparative research⁹⁸, and as such these participants may have been improperly matched and differences may be due to differences in training status. In line with the lack of difference in CS content, three studies have reported no sex differences in CS activity^{193–195}. In all of these studies VO2peak^{191–193} relative to fat-free mass was not different between the sexes^{193–195} and thus the finding that CS activity does not differ between males and females is quite plausible. Overall, a more careful examination of mitochondrial activity and function is required where males and females are matched for aerobic fitness in order to determine the effects of sex on oxidative metabolism.

It is important to once again point out that the lack of difference in the content of proteins related to lipid and oxidative metabolism may be due to the fact that fibre type did not differ between males and females. As noted, females have a greater proportion of type I muscle fibres¹⁹⁶. As type I fibres have a greater density of mitochondria, as well as are more oxidative in nature¹⁹⁶, it would then be conceivable that females should have a greater content of OXPHOS and CS protein content. In our current study, we report similar proportion of type I and type II muscle fibres between the sexes. Our reporting is slightly higher, but fairly similar to previous reports of a roughly 47% and 50% type I fibres in males and females respectively¹⁹⁷. This relatively similar fibre type composition of our male and female participants may then contribute to the lack of sex differences observed in content of the proteins examined. Thus, more

investigation is required to tease out sex-based differences in both content and activity of enzymes involved in lipid and oxidative metabolism in skeletal muscle, with careful consideration of matching for fitness and measuring fibre type

5.3 Strengths and Limitations

Alongside characterizing IMCL storage characteristics between the sexes, the intention of this project was to assess the effect of sex on IMCLs contribution to fuel utilization during an acute bout of HIIE. Main strengths of our study design include handling of the participants. Not only did we employ controls to ensure that males and females were matched based on physical fitness, but we used diet logs to determine nutrient intake to examine whether there were differences in habitual nutrient intake that may influence the results, and we controlled for both menstrual cycle and the use of birth control. These factors all contribute to ensuring that any of the differences in our results between the sexes were a result of inherent sex differences and not due to fluctuations due to daily life. Our study also used an exercise protocol widely used in healthy and clinical populations that can be used by the public with ease.

Our study does however, come with some limitations. While it was a major strength to account for dietary intake, this was completed through diet logs which is often accompanied by bias¹⁹⁸. As sex hormones such as estrogen and testosterone are derived from cholesterol¹⁹⁹, differences in unreported fat intake may have impacted our abilities to control for one of the many factors influencing sex hormones. Moreover, our results are only applicable to the follicular phase of the menstrual cycle as that is the phase in which we tested our female participants. Substrate oxidation is influenced by the menstrual cycle, whereby fat oxidation is greater in the luteal phase^{200–203}. Thus, had we done the trial in the luteal phase we may have seen greater differences in fuel utilization during the exercise bout between males and females.

Other limitations were due to the nature of the acute exercise bout. While RER values were used in the equations for %CHO and %fat oxidation, the original equations were validated for use with RQ¹⁴³. RER is only equal to RQ during rest and steady state exercise²⁰⁴. With our intervals at 90%VO₂peak, and lasting 1 minute, it is highly unlikely that steady state was reached, and our inferences of substrate oxidation are limited. Additionally, HIIE protocols often involve active recovery rather than total rest²⁰⁵. Thus, while following a classical HIIE design, the effects of sex on metabolism in our study are difficult to validate as the low intensity intervals were not at the same relative exercise intensity in males and females, skewing the females to an overall higher intensity. Also, our 5-minute cool down made it unlikely that enzyme activity determined from the post-exercise biopsy would match that seen during the high-intensity intervals, and limited our investigation of proteins to content, which is not necessarily indicative of activity. Ideally, the timing of the post-biopsy should change such that it is taken at the end of the last high intensity interval.

5.4 Future Directions

Further research is necessary to address sex differences in response to HIIE. As mentioned previously, estrogen is well evidenced to impact substrate utilization^{99,111,121,206}. These studies have largely used MIC exercise protocols^{121,207–210}, but whether its impact extends to HIIE must still further be examined. A continuance of this study to test females in other phases when estrogen is higher, such as during ovulation or the mid-luteal phase may reveal more pronounced differences.

Investigation of sex differences is important as females have been largely under-represented in sports and exercise medicine research thus far²¹¹. Such a sex disparity limits our current understanding of fuel utilization and benefits that may arise from novel modes of

exercise, such as HIIE. Furthermore, while we have been able to investigate an acute bout of HIIE, it would be valuable to implement a training program. This would provide better insight of the impact of sex and HIIE combined on factors that influence substrate utilization, such as lipid oxidation protein content and activity. This is especially notable as it has been shown that HIIT results in benefits to insulin sensitivity and glycemic control similarly to MIC training^{212,213}. However, there is a known sex difference to HIIT by which females have blunted improvements in insulin sensitivity compared to males^{2,50}. A mechanistic approach to investigation after training may be useful to make sex-specific training programs.

Furthermore, it has been well shown that MIC training influences IMCL content^{72,214–216}, localization^{67,69,217}, IMCL-and mitochondrial apposition^{27,79,91}. While we compared IMCL storage, localization and mitochondrial apposition between the sexes across an acute bout of HIIE, it would be valuable to investigate whether HIIT can elicit similar changes as MIC training over time, and whether there are sex differences that are uncovered over such a period. Additionally, while our study characterized morphological differences in IMCL storage, it would be beneficial to examine whether there is a sex difference in the profile of lipid species, as well as if there is a preferential usage of such lipids during both an acute bout of exercise and over training periods. We fibre typed using established methods of examining z-bands and m-lines¹⁴⁶, but other methods have combined IMF mitochondrial fraction with z-band width for fibre type classification^{174,218}. Doing so will also make possible an assessment of the proportion of mitochondria in contact with IMCL, which would provide valuable insights. Thus, our current study provides a starting basis to expand further into gaps of sex differences and metabolism.

5.5 Conclusions

The findings of this study add to a conflicting pool of evidence on lipids contribution to energy production during an acute bout of HIIE. Our findings support a lesser contribution of fats to energy production which is in line with previous reports with protocols similar to SIE. This finding may be attributable to our females working at an overall higher intensity during the exercise bout. Our findings of content of proteins largely involved in lipid mobilization and utilisation also showed no differences between the sexes, which may be better highlighted during other phases of the menstrual cycle and/or may be due to similarities in fibre type of our participants. Our findings highlight the importance of characterizing not only IMCL storage characteristics using electron microscopy, but IMCL morphology as well, as we noted differences in IMCL feret, perimeter, and perimeter to area ratio, suggestive of a greater opportunity for contact with mitochondria in females. Moreover, we also showed region specific sex differences and similarities for indicators of utilization such as change in the previously mentioned measures, as well as proportional contact, and number of mitochondria in contact with IMCL as indicators of extent of interaction. Future studies would benefit from a more direct measure such as stable isotope tracers in conjunction with indirect calorimetry, accounting for the demands of the low intensity exercise intervals, and either changing the timing of the post-exercise biopsy or adding a biopsy at the end of the last high intensity interval. Further investigation of TEM analyses of mitochondria might probe deeper into fibre-type specific differences,. Excitingly, the findings of the current study help to establish a new basis for characterizing IMCL morphology, storage and utilization, and to the best of our knowledge, is the first to perform this analysis in both males and females looking at sex differences.

Chapter Six: Significance of Research

The combination of positive changes to the cardiovascular system with HIIT^{4,219}, and evidence showing HIIE is more effective at lowering postprandial plasma triglyceride elevation than MICE²²⁰, warrants investigation into activation of aerobic systems and sources of lipid during HIIE. To the best of our knowledge, only one other group has begun to investigate lipid droplet morphology and length of contact with mitochondria in humans using TEM analyses^{147,221}, but only in clinical populations and omitted females. Thus our findings ameliorate the underrepresentation of females as subjects in exercise science²¹¹, which is increasingly prominent in studies regarding interval training²²². Indeed, it has been noted that research into skeletal muscle mitochondrial responses of interval training in women is relatively restricted and discordant^{222–226}. By building upon physiological differences at baseline between the sexes^{15,28,227}, we are able to provide a foundation for narrowing this gap in our understanding. Interestingly, our reported difference in relative work rates between the sexes during the low-intensity intervals may aid our understanding of differential responses to the same protocol. This finding may help to better prescribe exercise in consideration of relative intensities during both work efforts and rest, as to not mask sex differences with differences in metabolic demands of the exercise bout itself. Our study provides novel findings that establish a basis for future research to be conducted on IMCL morphology, localization, and interaction with mitochondria, as well as to set guidelines on ‘levelling’ the playing-field of intensities so that valuable insights on mechanisms underlying sex differences in interval training can be made.

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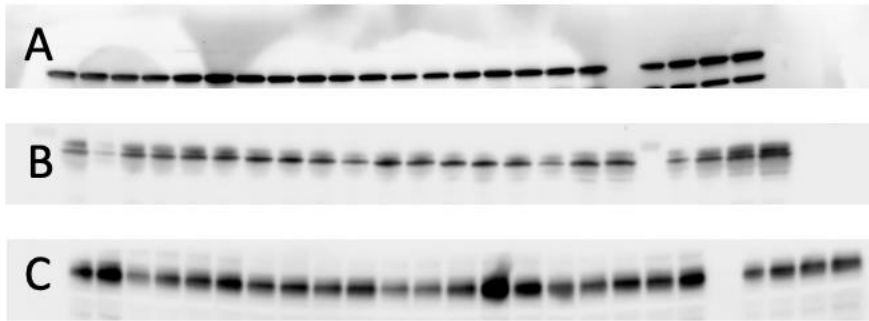
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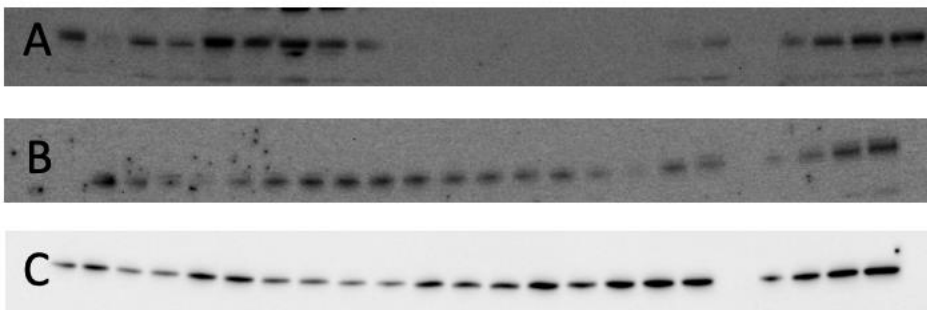
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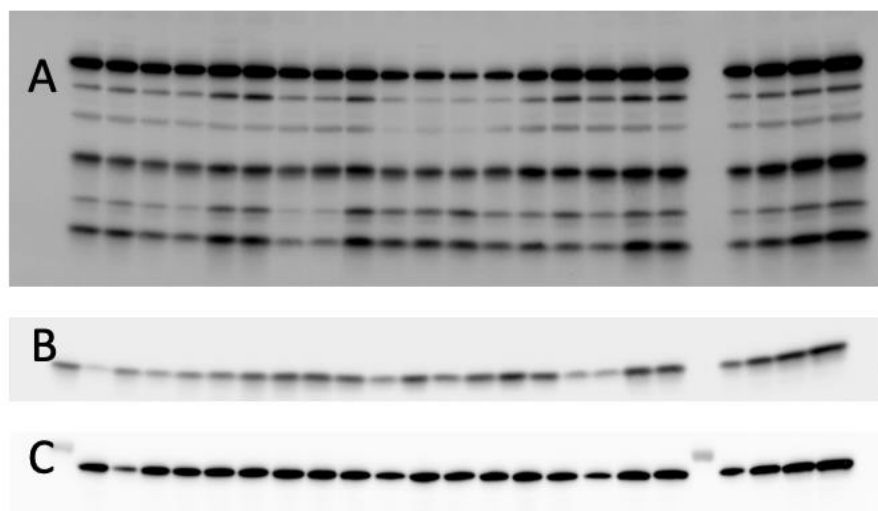
Appendix A



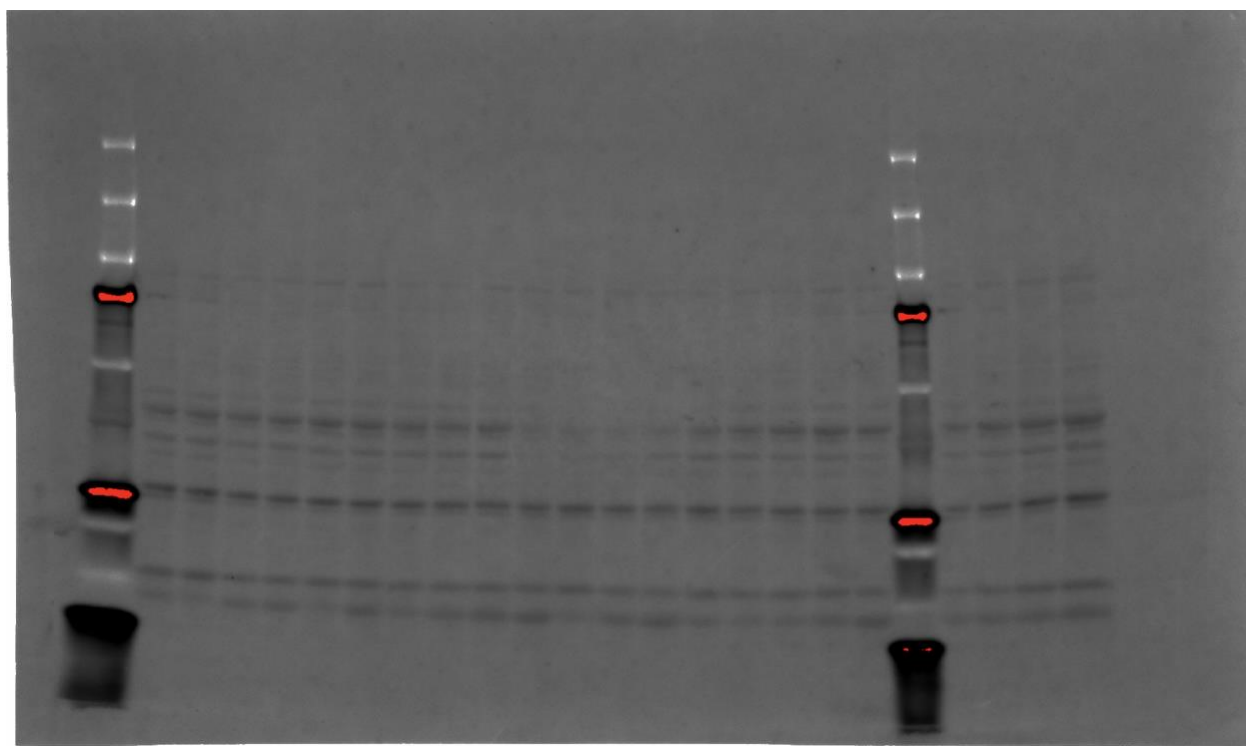
Supplementary Figure 1. Representative whole-blots for PLIN2, PLIN3, and PLIN5. Loading scheme followed the pattern of female (pre, post), then male (pre, post) and alternated. The first and seventh participants samples were re-run on a subsequent gel. The four right-most bands are the standard curve.



Supplementary Figure 2. Representative whole-blots for ATGL, CPT1, and SCHAD.. Loading scheme followed the pattern of female (pre, post), then male (pre, post) and alternated. The first and onwards of the fourth participants were re-run on subsequent gels. The four right-most bands are the standard curve.



Supplementary Figure 3. Representative whole-blot for Complex I-V cocktail, Complex IV+IVL2, and CS. Loading scheme followed the pattern of female (pre, post), then male (pre, post) and alternated. From the top of A) bands 1, 2, 4, 5 and 6 correspond to Complex V, Complex III, Complex II, Complex IV, and Complex I, respectively. The four right-most bands are the standard curve.



Supplementary Figure 3. Representative Stain-Free Blot representing total protein content to be normalized to. Lanes 1 and 22 are ladders, lanes 2-19 are individual subject samples, and wells 23-26 are standard curves.